Hybridisation and Its Consequences

Population Genomics, Herbivory, and Phytochemistry in the *Senecio nemorensis* Syngameon

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Manuela Bog

dedicated to my parents
and my friend

Previous page: *Senecio hercynicus*-like and *Senecio ovatus*-like individuals near Oberwiesenthal, Ore Mountains, Saxony (Germany)

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General Abstract

Hybridisation often determines the evolutionary trajectories of closely related species and may lead on the one hand to the formation of new species or on the other hand to a loss of species diversity through genetic swamping or fusion of taxa. The three Central European representatives of the *Senecio nemorensis* syngameon, *S. germanicus*, *S. ovatus*, and *S. hercynicus* are vertically vicariant species that hybridise in their range overlaps. In the present dissertation the following three key aspects concerning consequences of hybridisation among the three species are highlighted. Firstly, by using a population genomic approach, the extent of hybridisation between *S. hercynicus* and *S. ovatus* and possible natural selection regimes are investigated along four elevational transects in the Bavarian Forest National Park (Bavaria, Germany). Secondly, transplantation experiments were performed to test for differences in consumption of pure species and their artificial F_1 hybrids by herbivores along an elevational gradient. Finally, in food choice experiments the observed results from the field were checked under controlled lab conditions and a possible relationship between consumption and chemical defence compounds (i.e., pyrrolizidine alkaloids and tannins) was investigated. Addressing the first key aspect, we found advanced introgressive hybridisation along the whole transects. Most individuals could be assigned to the *S. ovatus* backcross class and just very few *S. hercynicus*-like genotypes were present. Although evidence was found for a climate-mediated divergent selection, this force is interpreted as not being strong enough to maintain the two biological species. During the transplantation experiment it was shown that *S. hercynicus* also suffers to a higher extent from herbivory than *S. ovatus* but there was also evidence for an elevated susceptibility to low temperatures of the latter at elevations typical for *S. hercynicus*. In contrast, *S. germanicus* showed low herbivore damage relative to *S. hercynicus* and low leaf area loss through withering relative to *S. ovatus*. In the food choice experiments, the results from the field could be proven to some extent. It was shown that as soon as four leaf discs of the different taxa (i.e., parental species and reciprocal hybrids) were offered to the molluscs, the selective consumption found for the pure taxa was compensated. Hybrids

were neither more susceptible nor more resistant than their corresponding parental species. A correlation of feeding preference and content of chemical defence compounds could only partly be proven for pyrrolizidine alkaloids. Tannins had no effect at all on the consumption by molluscs. To conclude, all results from the present studies indicate that *S. hercynicus* will lose to *S. ovatus* in the Bavarian Forest and neither climate- nor herbivory-mediated selection seems strong enough to keep both species as divergent lineages. In the *S. germanicus*-*S. ovatus* system the evolutionary trajectories are not assumed to lead to an extinction of either species. Rather, a patchily distribution of pure and hybrid populations is expected from the results.

CHAPTER 1

Introduction

1.1 Hybridisation

Hybridisation is a naturally occurring and evolutionary important process that is known from both plants (Arnold et al., 1990; Marcussen et al., 2001; Oberprieler et al., 2013; Salvini et al., 2009) and animals (Araya-Anchetta et al., 2013; Daguin et al., 2001; Seehausen et al., 2008). About 25% of plant species and 10% of animal species are involved in hybridisation (Mallet, 2005). While on the one hand, hybridisation is seen as destructive process, leading to extinction of species (Balao et al., 2015; Vonlanthen et al., 2012) and therefore loss of biodiversity, it may lead on the other hand to new adaptive traits and even to the formation of new species (Mallet, 2007). In addition, there are also studies known where hybridisation leads to stable hybrid zones that neither result in the extinction of species nor in the formation of new hybrid species as it is the case for example in *Picea* trees (De La Torre et al., 2014) or *Heliconius* butterflies (Rosser et al., 2014). The formation of stable hybrid zones depends on the fitness of the hybrid genotypes in comparison to the parental ones. The 'tension zone model' (Barton and Hewitt, 1985) predicts that hybrids are less fit than the parental genotypes due to intrinsic selection. Subsequently, this hybrid zone is maintained by a balance of intrinsic selection against the hybrids and their dispersal (Abbott and Brennan, 2014). The 'bounded hybrid superiority model' (Moore, 1977) predicts that hybrids are fitter than the parental genotypes in the transition zone, but less fit in the parental habitats. In this zone extrinsic selection provides an advantage for the hybrids in intermediate environmental habitats (Abbott and Brennan, 2014). Extrinsic selection can be mediated by abiotic factors like climate as well as by biotic factors like herbivory and competition. Climate-mediated selection

and its consequences for two hybridising plant species of the genus *Senecio* L. (Compositae, Senecioneae) will be in focus of the first part of the present dissertation, while the second part will investigate how herbivory influences the evolutionary trajectories of hybrid and parental genotypes.

1.2 The genus *Senecio* L.

The genus *Senecio* L. (Compositae) is one of the largest genera in the flowering plants. It consists of c. 1,250 species with an almost cosmopolitan distribution (Bremer and Anderberg, 1994; Coleman et al., 2003; Jeffrey, 1986, 1992; Nordenstam, 1978, 2007). They are very variable in life-history strategies and morphology. There are annual or perennial herbs, shrubs, vines, trees and epiphytes (Pelser et al., 2007). A further characteristic of this genus is the presence of pyrrolizidine alkaloids (PAs) of the senecionine type and the presence of eremophilane and furanoeremophilane sesquiterpenes (Blaschek et al., 2007). These phytochemical compounds play an important role in herbivore deterrence as they are reported to be toxic to humans and many vertebrates and invertebrates (Candrian et al., 1984; Dreger et al., 2009; Grecco et al., 2011; Mattocks, 1986). However, toxicity depends on the animal species and can be very different (Cheeke, 1988). Additionally, there are several examples known, were especially insects adapted to the PAs in plants using them for their own protection or as pheromone precursors (Dobler et al., 2000; Edgar et al., 1976; Hartmann, 1999).

There are many examples that hybridisation plays an important role in the genus *Senecio* (Chapman and Jones, 1971; Kadereit et al., 2006; López et al., 2008; Prentis et al., 2007). A well studied hybrid system is represented by the *S. chrysanthemifolius-S. aethnensis* hybrid zone on Mt. Etna (Sicily, Italy) with *S. chrysanthemifolius* growing in lower and *S. aethnensis* in higher elevations. Hybrids can be found at intermediate elevations (Brennan et al., 2009). As there is evidence that strong intrinsic selection is acting against the hybrids, this zone was characterised as a 'tension zone' (Abbott and Brennan, 2014). However, the hybrids gave rise to *S. squalidus*, a highly successful homoploid hybrid species from a further cross with *S. vulgaris* in Great Britain (James and Abbott, 2005). A second well studied hybrid system is the *Jacobaea vulgaris-J. aquatica* system (formerly *S. jacobaea* and *S. aquatica*). In this system, a main focus of research lies on the influence of hybridisation on secondary metabolite composition, particularly of PAs, and its effects on herbivory. Cheng et al. (2011a) could show that the observed PA variation was greater in hybrids than in parental plants and partially novel compositions were expressed in F_2 individuals. Feeding damage by a generalist

insect herbivore *(Frankliniella occidentalis)* decreased with increasing jacobine-like PA concentration in the plants (Cheng et al., 2011c). Additionally, Wei et al. (2015) found a negative correlation of feeding damage with total PA concentration by the generalist slug species *Deroceras invadens*. In contrast, the damage by *Longitarsus jacobaeae*, a specialist flea beetle, was not influenced by PA content (Wei et al., 2015).

Each of the two study systems shows that *Senecio* provides an interesting genus to study evolutionary processes, especially hybridisation and its consequences for natural selection, adaptation, phytochemistry and herbivory. As the *Senecio nemorensis* syngameon is in focus of the present dissertation, the next section will give a short overview on this group and what is already known from previous studies on hybridisation and herbivory, particularly for the three Central European representatives.

1.3 The *Senecio nemorensis* syngameon

The *S. nemorensis* group consists of nine species with a couple of subspecies and varieties (Table 1.1). They are distributed throughout Europe and Asia, ranging from Portugal to Japan and from Murmansk (Russia) to Taiwan (Herborg, 1987). As shown in Figure 1.1, hybridisation in this group is a common phenomenon and various hybrids connect several species (Herborg, 1987; Oberprieler, 1994, 2010, 2011; Hodálová and Marhold, 1996, 1999). Hence, one is also speaking of a so called syngameon (Lotsy, 1925, 1931; Grant, 1981). The isolation barriers depend either on geographical and elevational distribution and/or differences in phenology, but up to now, they are not known to be postzygotically determined. Thus, the formation of fertile F_1 hybrids often leads to backcrosses with the parental species, introgressive hybridisation and to the formation of hybrid swarms in overlapping regions (Oberprieler et al., 2010, 2011, 2015).

In 1987, Herborg wrote a comprehensive monograph on the *S. nemorensis* syngameon, in which he already described the existence of hybrids among several species from this group. Just a few years later, Oberprieler (1989,1994) surveyed different *Senecio* populations in Bavaria (Germany) and could prove the observations by Herborg (1987) concerning hybridisation based on multivariate analyses of several morphological characters. Using molecular genetic methods (RAPD fingerprinting), Raudnitschka et al. (2007) investigated populations of *S. ovatus* and *S. hercynicus* along an elevational gradient on Mt. Brocken (Harz Mountains, Anhalt-Saxony, Germany). They found that the strength of introgressive hybridisation depends on the elevation, with *S. hercynicus* occurring at high elevations, *S. ovatus* occurring at low elevations,

Table 1.1: List of taxa included in the *Senecio nemorensis* syngameon following Herborg (1987), modified according to Hodálová and Marhold (1998), and Hodálová (1999)

Senecio bayonnensis Boiss.
Senecio cacaliaster Lam.
Senecio dacicus Hodálová & Marhold
Senecio germanicus Wall.
subsp. *germanicus*
var. *germanicus*
var. *karaulensis* (Form.) Herborg
subsp. *glabratus* Herborg
Senecio hercynicus Herborg
subsp. *durmitorensis* Herborg
subsp. *hercynicus*
var. *expansus* (Boiss. & Heldr.) Herborg
var. *hercynicus*
Senecio nemorensis L.
Senecio ovatus (P.Gaertn. Mey. & Scherb.) Willd.
subsp. *alpestris* (Gaudin) Herborg
subsp. *ovatus*
Senecio stabianus Lacaita
Senecio ucranicus Hodálová

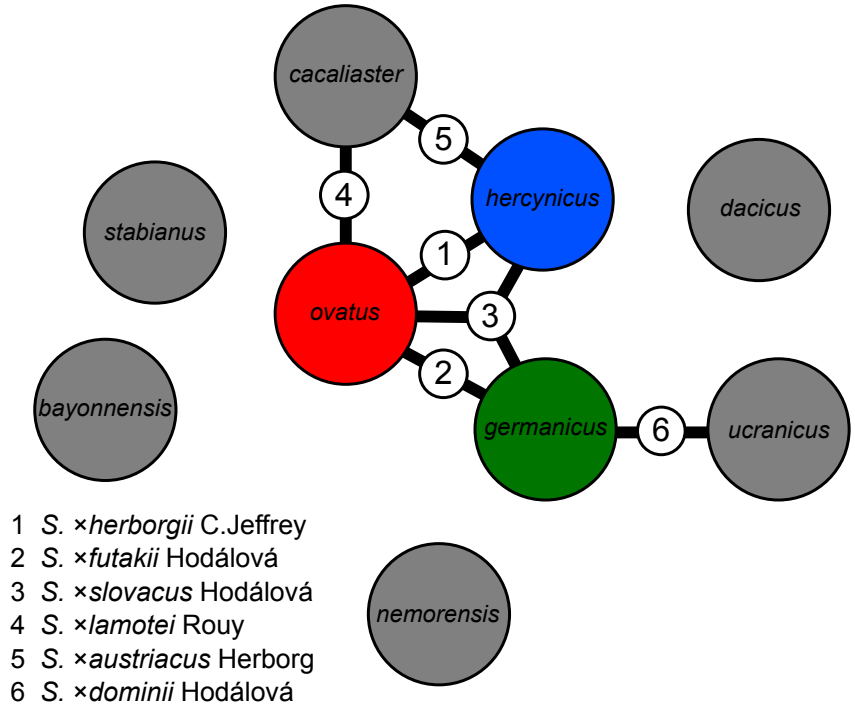

Figure 1.1: The *Senecio nemorensis* syngameon. Numbered lines indicate among which species hybridisation is known.

and hybrids occurring at intermediate elevations. The investigation of a single hybrid swarm on S- and SW-facing slopes of the German part of the Bohemian Forest by morphological characters and AFLP fingerprinting revealed that the majority of individuals were found to be intermediate between the two parental species (*S. hercynicus* and *S. ovatus*). In addition a strong correlation between phenetic and genetic distances was found (Oberprieler et al., 2010). A phytochemical survey of pyrrolizidine alkaloids in the same population revealed that the investigated individuals fell into one of two main chemotypes. Only few individuals exhibiting intermediate or additive PA patterns were found (Oberprieler et al., 2010). Furthermore, a survey by Oberprieler et al. (submitted) concerning herbivory showed that the feeding damage by herbivores, predominantly molluscs, was significantly influenced by the genetic constitution (*S. hercynicus*-like individuals were stronger damaged than hybrid or *S. ovatus*-like individuals), by phenology (early-flowering individuals were stronger damaged than late flowering ones), and by PA concentration (*S. hercynicus*-like individuals were increasingly damaged with increasing PA content and hybrid/*S. ovatus*-like individuals were decreasingly damaged with increasing PA content). In the same study, it was shown that pollen fertility, seed set and germination rate was not reduced in natural hybrid individuals.

Additionally, Oberprieler et al. (2011) investigated 15 populations of *S. germanicus* and *S. ovatus* north of Regensburg (Germany). Multivariate analyses based on morphological characters as well as AFLP fingerprint data showed that the two species formed distinct entities and did not hybridise with each other. However, in one population from the Upper Palatinate Forest, a high number of hybrid individuals were found, that were identified as backcrosses towards *S. germanicus*. Here, phytochemical analyses revealed that hybrids exhibited an additive PA pattern or a pattern similar to *S. germanicus*, while in quantitative respects all hybrids were similar to *S. germanicus*. A recent publication by Oberprieler et al. (2015) surveyed eleven populations of *S. hercynicus* and *S. ovatus* along an elevational transect in the Bavarian Forest National Park (Bavaria, Germany) that were identified as being hybrid swarms with different admixture proportions of both parental genotypes. Only few AFLP markers were found as being under selection, with some correlated to climatic variables. However, none of these markers was species-specific.

The results of the aforementioned studies show that hybridisation has a strong influence on the evolutionary trajectories of the studied species, which seem to follow different paths in the two hybrid systems under study (i.e., the *S. hercynicus-S. ovatus* system and the *S. germanicus-S. ovatus* system). Again, this provides a perfect starting point for further investigations concerning hybridisation and its consequences.

1.4 Study objects

The study objects of the present dissertation, *S. germanicus* Wallr. subsp. *germanicus* var. *germanicus* (henceforth referred to as *S. germanicus*), *S. hercynicus* Herborg subsp. *hercynicus* var. *hercynicus* (henceforth referred to as *S. hercynicus*), and *S. ovatus* (P.Gaertn., Mey. & Scherb.) Willd. subsp. *ovatus* (henceforth referred to as *S. ovatus*) are tetraploid ($2n = 40$), insect-pollinated, perennial geophytes and reported to be self-incompatible (Herborg, 1987; pers. obs. 2011). They are capable of producing large numbers of wind-dispersed fruits (achenes) and also propagate vegetatively by formation of stolones except *S. germanicus*. All three are members of plant communities typical of succession stages after natural or anthropogenic disturbances (e.g., forest clearings). Conspicuous differences in morphology between the study taxa concern the leaf bases of the upper cauline leaves, which are sessile in *S. germanicus* and *S. hercynicus* and petiolate in *S. ovatus*. Further, the dimension and shape of the capitula is discriminating the three taxa. *Senecio germanicus* and *S. hercynicus* are characterised by larger capitula with a higher number of tubular/disc florets and involucral bracts than *S. ovatus*. In

Figure 1.2: Habitus and close-up views of stem and capitulum of *Senecio hercynicus* (upper row), *S. ovatus* (mid row) and *S. germanicus* (lower row).

addition, in *S. germanicus* the supplementary involucral bracts are furnished with long eglandular hairs and also the lower part of the stem possesses long eglandular hairs. *Senecio hercynicus* is characterised by the possession of

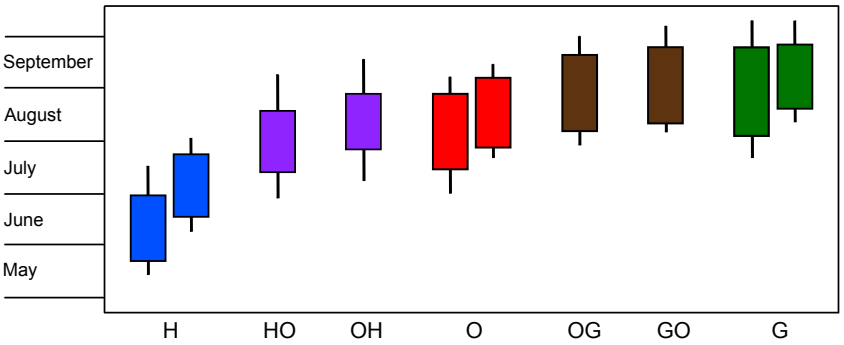

Figure 1.3: Flowering time of *S. hercynicus, S. ovatus, S. germanicus* and their reciprocal F_1 hybrids. For the pure species flowering time was measured in 2011 (first bar) and 2013 (second bar). F_1 hybrids were measured in 2013. Box indicates the mean date, when the first capitulum started to flower until the all capitula were faded. Error bars indicate standard deviations.

glandular hairs on leaves, the upper part of the stem and on the supplementary bracts of the capitulum. In contrast to these two species, in *S. ovatus* only articulate hairs are found. Figure 1.2 shows the habitus and the most conspicuous morphological traits of the three study species. When growing in a common-garden experiment at the University of Regensburg (Regensburg, Bavaria, Germany), the three species exhibit considerable differences in phenology: although the shoots emerge nearly simultaneously around the middle of April, *S. hercynicus* grows much faster than the other two species and flowers about four weeks earlier than *S. ovatus*, while *S. ovatus* in turn flowers about two weeks earlier than *S. germanicus*. The average flowering time of *S. hercynicus* is in general between June and July, for *S. ovatus* between July and August and for *S. germanicus* between July and September. However, isolation by flowering time is not complete and hybridisation is possible, with corresponding F_1 hybrids showing intermediate flowering times (Figure 1.3). The distribution of the three study species throughout Central and South East Europe is shown in Figure 1.4. A further characteristic is their vertical vicariance, with preferences of lower elevations and more continental climatic conditions by *S. germanicus*, intermediate ones with more oceanic conditions by *S. ovatus*, and high elevations with a high montane or subalpine climate by *S. hercynicus*.

1.5 Thesis outline

The aim of the present dissertation is to investigate the consequences of hybridisation on three representatives of the *Senecio nemorensis* syngameon,

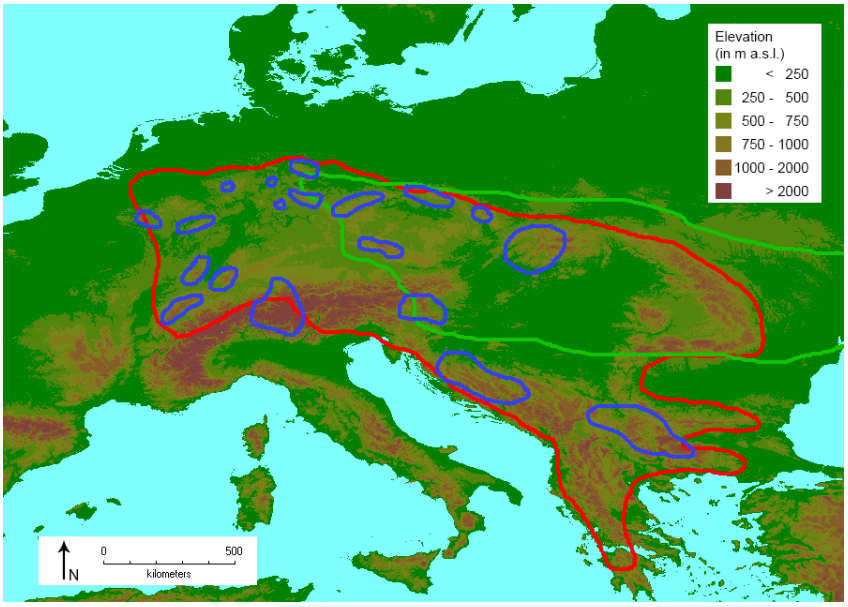

Figure 1.4: Distribution of *S. germanicus* subsp. *germanicus* var. *germanicus* (green line), *S. ovatus* subsp. *ovatus* (red line) and *S. hercynicus* subsp. *hercynicus* var. *hercynicus* (blue line) throughout Central and South-East Europe according to Oberprieler (1994).

in particular *S. germanicus*, *S. hercynicus* and *S. ovatus*. In chapter 2, the extent of hybridisation between *S. hercynicus* and *S. ovatus* is studied using a population genomic approach. Additionally, it is investigated whether there is a climate-mediated selection regime acting that maintains the two biological species. Chapter 3 reports on the results of transplantation experiments of pure parental species and their artificial F_1 hybrids that were conducted along an elevational gradient. Here, the main focus lies on differences in consumed leaf area by herbivores, particularly molluscs, but also other parameters like active photosynthetic leaf area, withered leaf area and rhizome dry weight are considered. In chapter 4, the pure parental species and their artificial F_1 hybrids are investigated for their qualitative and quantitative differences in phytochemical compounds that are known to be deterrent on herbivores. Additionally, selective consumption among the parental and hybrid taxa is investigated by two mollusc species in food choice experiments under controlled lab conditions. Moreover, the correlation between deterrent compounds and consumption of the taxa is examined. Finally, chapter 5 gives a general discussion on the findings in this dissertation and will outline potential experiments that could be done to further complete the understanding of hybridisation and its consequences in the present study system.

CHAPTER 2

Population genomic investigations of the two hybridising species *Senecio hercynicus* and *S. ovatus* (Compositae, Senecioneae) along elevational transects in the Bavarian Forest National Park

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Author contributions: MB, CB, and CO conceived and designed the experiments. MB performed the experiments. MB analysed the data. MB wrote the draft.

2.1 Abstract

Hybridisation is an important evolutionary process. The investigation of hybridisation along elevational transects, with their steep abiotic and biotic clines, provides insight into the adaptation and maintenance of species in adjacent habitats. *Senecio ovatus* and *S. hercynicus* are vertically vicariant species that show hybridisation in their range overlaps. In the present study, we report on a population-genomic approach using AFLP fingerprinting of 689 individuals from 38 populations along four elevational transects (650-1350 m) in the Bavarian Forest National Park. A maximum-likelihood based hybrid index shows that the overall genetic differentiation among all populations is very low. Almost 75% of the investigated individuals were classified as backcrosses towards *S. ovatus,* and 25% were classified as belonging to the F_1/F_2 classes. There were just a few backcross individuals towards *S. hercynicus* and a vanishingly small number of pure parental individuals. The highest *S. hercynicus* ancestry was found in the uppermost populations of two transects. A genome scan method revealed 47 loci that deviated from a model of neutral introgression. Finally, the search for loci under divergent selection detected some loci that correlated with climate variables concerning precipitation and temperature. According to our results, hybridisation of *S. ovatus* and *S. hercynicus* has reached an advanced state of genetic swamping in the Bavarian Forest National Park and there seems to be no driving force that is strong enough to keep both species as different lineages.

2.2 Introduction

Hybridisation is considered to be a 'double-edged sword' in conservation biology (Haig and Allendorf, 2006) as it may lead on the one hand to the formation of new species (Abbott et al., 2013; Grant and Grant, 1998), and on the other hand to the extinction of species through fusion of two genetic differentiated taxa or genetic swamping of one taxon by another (Levin et al., 1996; Rhymer and Simberloff, 1996). Besides these rather extreme outcomes of hybridisation, stable hybrid zones may occur, where no progress towards speciation is made, but also existing differentiation between species is maintained (Abbott et al., 2013). Stable hybrid zones are described by the (1) 'tension zone model' and by the (2) 'bounded hybrid superiority model'. In the first case, hybrids are less fit than the parental species and the hybrid zone is maintained by a balance of environment independent selection (intrinsic selection) against the hybrid genotypes and their dispersal (Barton and Hewitt, 1985; Abbott and Brennan, 2014). In the second case, hybrids are fitter than the parental species in transition zones but less fit in parental habitats (Moore, 1977). Here, selection is environment dependent (extrinsic selection) and hybrid genotypes occupy intermediate environmental zones (Abbott and Brennan, 2014). In a situation, where hybrid genotypes exhibit a higher fitness in one parental genotypes habitat, but lower fitness in the other, the hybrid zone should move until the balance of selection and dispersal is reached as predicted under the 'bounded hybrid superiority model' or until the inferior species is eliminated and replaced by the other (Fritz, 2001).

Elevational transects, with their steep clines of abiotic (temperature, moisture, hours of sunshine, UV radiation, season length, geology; Körner, 2007) and biotic conditions (pollinator and herbivore communities and densities), provide excellent opportunities to gain insight into the adaptation and maintenance of species in adjacent habitats in the presence of hybridisation and therefore became interesting study systems (Abbott and Brennan, 2014). Quite recently, Abbott and Brennan (2014) reviewed twelve plant hybrid zones occurring across elevational gradients that were under molecular genetic study within the last 25 years. As in the case of plant groups involved, ranging from trees *(Picea, Pinus, Populus, Quercus)* and shrubs *(Artemisia, Rhododendron)* to herbaceous perennials *(Aquilegia, Ipomopsis, Penstemon, Senecio, Silene),* geographical locations (North and Central America, Europe, and Asia) were diverse (Abbott and Brennan, 2014; and references therein). Since not all hybrid zones were studied comprehensively (e.g., comprising reciprocal transplantation experiments or lifetime fitness evaluations), classification into one of the hybrid zone models was not possible and remained unsatisfactory in many cases (Abbott and Brennan, 2014). Amongst others, the authors mentioned

the example from the *Senecio nemorensis* syngameon in their review, which is also in focus in the present study.

Senecio hercynicus Herborg subsp. *hercynicus* var. *hercynicus* and *S. ovatus* (P.Gaertn., May. & Scherb.) Willd. subsp. *ovatus* are vertically vicariant species. Due to its broader ecological amplitude *S. ovatus* inhabits elevations between the colline and the montane belts and shows a continuous distributional range throughout central and south-eastern Europe. *Senecio hercynicus,* with a disjunct distribution, grows only in the montane to subalpine belts of the Central European highlands, parts of the Alps, and the south-eastern European mountains, leading to mixed stands of the two species at intermediate elevations. The tetraploid ($2n = 40$), insect-pollinated, perennial geophytes are reported to be self-incompatible (Herborg, 1987; pers. obs. 2011). They are capable of producing large numbers of wind-dispersed fruits (achenes) and also propagate vegetatively by formation of stolones. Both are members of plant communities typical of succession stages after natural or anthropogenic disturbances (e.g., forest clearings). The distribution ranges of the two species overlap in most parts of central and south-eastern Europe. Conspicuous differences in morphology between the study taxa concern the leaf bases of the upper cauline leaves, which are sessile in *S. hercynicus* and petiolate in *S. ovatus.* Further, the dimension and shape of the capitula is diagnostic. *Senecio hercynicus* is characterised by larger capitula with a higher number of tubular/disc florets and involucral bracts than *S. ovatus.* In addition, *S. hercynicus* is characterised by the possession of glandular hairs on leaves, the upper part of the stem and on the supplementary bracts of the capitulum. In *S. ovatus* only articulate hairs are found. When growing in mixed stands in nature or being cultivated in a common-garden experiment, the two species exhibit considerable differences in phenology (pers. obs. 2011, 2013). Although the shoots emerge nearly simultaneously around the middle of April, *S. hercynicus* grows much faster than *S. ovatus* and flowers about five weeks earlier. The average flowering time of *S. hercynicus* is in general between June and August and for *S. ovatus* between July and September (Dierschke, 1995). However, isolation by flowering time is not complete, which makes hybridisation possible.

Studies in mixed stands of *S. hercynicus* and *S. ovatus,* especially in Bavaria (Germany), revealed that hybridisation between the two species is rather the rule than an exception. On the basis of morphological traits, Oberprieler (1994) found only a single population out of seven surveyed, where both species occurred next to each other without signs of hybridisation. In a further study in the Bohemian Forest, Bavaria (Germany), morphological and genetic analyses (AFLP fingerprinting) indicated, that the investigated mixed stand formed an intensively introgressed hybrid swarm, where the majority of individuals

occupied an intermediate position between the parental species (Oberprieler et al., 2010). This raised the question to what extent hybridisation occurs between the two vertically vicariant species along an elevational transect and whether divergent selection could prevent genetic swamping of *S. hercynicus* by *S. ovatus.* In a population-genomic approach, which was successfully applied following the methodology described by Herrera and Bazaga (2008) and Holt et al. (2011), Oberprieler et al. (2015) found hybrid swarms with different admixture proportions of both parental genotypes along the surveyed transect in the Bavarian Forest National Park. Additionally, because of the lack of species-specific AFLP markers under positive selection the authors conclude that there is no evidence for a climatically mediated divergent selection or a reinforcement process, unavoidably leading to the genetic swamping of *S. hercynicus* by *S. ovatus.*

The present study is a continuation of the survey by Oberprieler et al. (2015). We here investigate four elevational transects in the Bavarian Forest National Park (including the afore mentioned transect) to check whether these preliminary results correspond to a general pattern in the whole range of the national park. For that purpose, we optimised the AFLP protocol of Oberprieler et al. (2015) to get more reliable banding patterns (reduction of fragments and background noise). Additionally, we used two known pure stands of *S. hercynicus* and *S. ovatus* for calibration in subsequent analyses. The following questions are therefore in the focus of the present study. (1) What is the extent of hybridisation between *S. hercynicus* and *S. ovatus* along four elevational transects in the Bavarian Forest National Park? (2) Are there loci under divergent selection that correlate with climatic variables? (3) Are there species-specific loci that deviate from a model of neutral introgression, i.e., loci that show increased or decreased introgression rates that could be evidence for loci that are linked to genes, which are advantageous in a certain habitat, or loci that are linked to reproductive isolation, respectively?

2.3 Material and methods

2.3.1 Plant material and DNA extraction

Plant material was collected along four elevational transects 'Plattenhauser' (A), 'Rachel' (B), 'Scheuereck' (C) and 'Lackenberg' (D) (Figure 2.1) in the Bavarian Forest National Park (SE Germany). They reach from montane elevations of 600 m to high-montane ones above 1300 m and were set up by the BIOKLIM project (Bässler et al., 2009; 2010) to monitor long-term biodiversity changes caused by climatic changes. At each reference point of the transects,

which were established at distances of 100 m, *Senecio* stands in close vicinity (30 m) around the reference points were considered and 10-20 individuals per stand randomly sampled for leaf material. After drying and storing in silica gel, total genomic DNA for a total of 689 plants from 38 populations (A: 191/11, B: 151/8, C: 133/7, D: 214/12) along the transects (Figure 2.2; Table 2.1) was extracted according to a modified protocol of Rogstad (2003) based on silica (detailed protocol in Oberprieler et al., 2013).

Owing to the lack of pure populations of the parental species in the region of our study, we included 19 DNA samples from the geographically closest candidate reference population of *S. hercynicus* (H) from about 250 km SW in the Allgäu region (Bavaria, Germany) (Oberprieler, 1994) and 19 samples from a reference population of *S. ovatus* (O) from about 12 km ENE from Regensburg (Bavaria, Germany) assuming that these populations presumably looking back onto a long, independent history of diversification.

2.3.2 AFLP fingerprinting

AFLP fingerprinting was performed following the protocol of Vos et al. (1995) with some modifications. Sixty-three individuals (about 9% of the total number of OTUs) were replicated to assess the reproducibility of fragments. For the combined restriction-ligation reaction, 100 ng DNA was incubated at 37°C for 3.5 h, including 2.5 U of each *Vsp*I and *Eco*RI, 0.5 U T4 DNA ligase, 1x restriction buffer O, 1x ligation buffer including 5 mM ATP (all Fermentas, St. Leon-Rot, Germany), as well as 1 µM and 0.1 µM of the original *Mse*I and *Eco*RI adaptors, respectively, in a total reaction volume of 10 µl. A pre-selective PCR was carried out in 5 µl containing 1 µl diluted (1:10) template DNA from the restriction–ligation step, 3.75 µl AFLP CoreMix (Applied Biosystems, Carlsbad, USA), and 0.5 µM of primers with one selective nucleotide (*Vsp*I-C 5' – GATGAGTCCTGAGTAATC – 3' and *Eco*RI-A 5' – GACTGCGTACCAATTCA – 3'). The selective PCRs were carried out in 5 µl containing 0.75 µl diluted (1:20) template DNA from the preselective step, 0.125 U Taq polymerase, 1x reaction buffer S, 0.2 mM of each of the dNTPs (all PeqLab, Erlangen, Germany), and primers with three selective nucleotides (0.1 µM of the selective *Eco*RI-ANN primer and 0.5 µM of the selective *Vsp*I-CNN primer). Both PCR cycling protocols were adopted from Meister et al. (2006). For each sample, three selective PCRs with differently fluorescence-labelled *Eco*RI primers and the following three primer combinations were conducted: (D2) *Eco*RI-AAC/ *Vsp*I-CTA, (D3) *Eco*RI-ACA/ *Vsp*I-CTT, (D4) *Eco*RI-ACA/ *Vsp*I-CTA. The PCR products were multiplexed, precipitated, and subsequently dissolved in a mixture of GenomeLab Sample Loading Solution and CEQ Size Standard 400 (both Beckman Coulter, Fullerton,

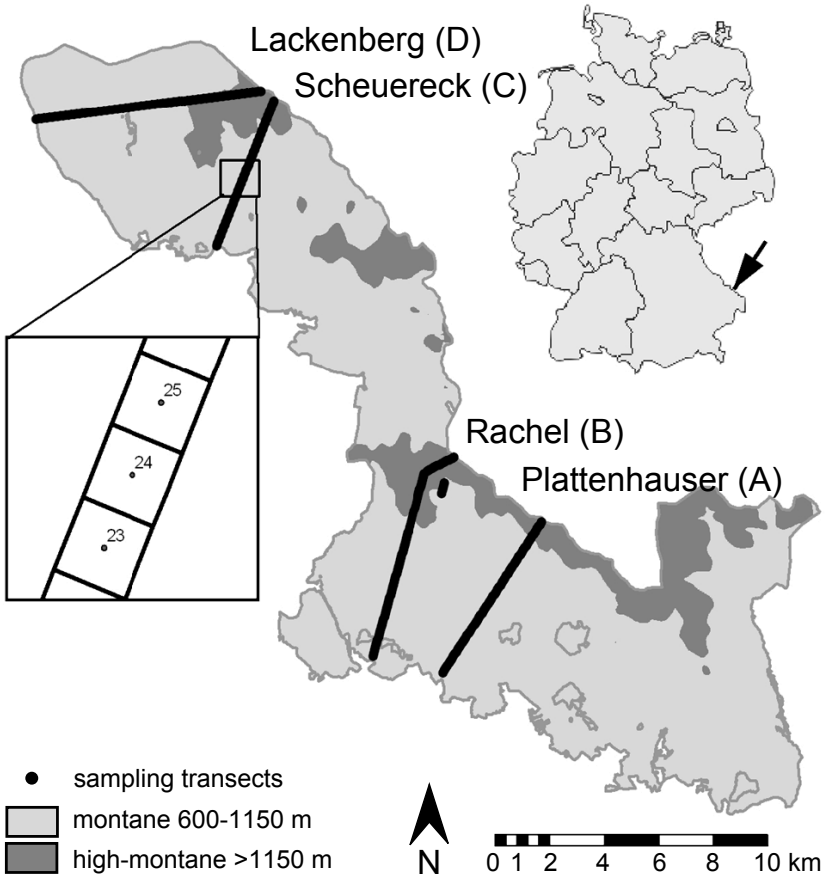

Figure 2.1: Geographical location of the four investigated transects (black lines) in the Bavarian Forest National Park and its location in Germany (small overview map). Adapted from Bässler et al. (2009).

USA). Fragment detection was performed on a CEQ8000 capillary sequencer (Beckman Coulter, Fullerton, USA).

AFLP data were automatically analysed and scored into a 0/1 matrix with GelCompare II vers. 5.1 (Applied Maths Inc., Austin, TX, USA). To determine optimal scoring parameter settings, a modified protocol of Holland et al. (2008) was used. The following parameters were tested to determine the best scoring parameter settings: Firstly, we varied the minimum profiling (percents relative to the maximum value of the lane: 0.10%, 0.25%, 0.50%, 0.75%, 1.00%, 1.50% or 2.00%), the minimum area (0.0%, 0.1%, 0.2% or 0.3%) and the shoulder sensibility (0, 5) in the autosearch band tool. Secondly, we varied the position tolerance in the band matching tool (0.03%, 0.06%, 0.10% and 0.13%; with 1 bp length difference equivalent to an average 0.10% length difference). Additionally, we tested two different fragment length ranges

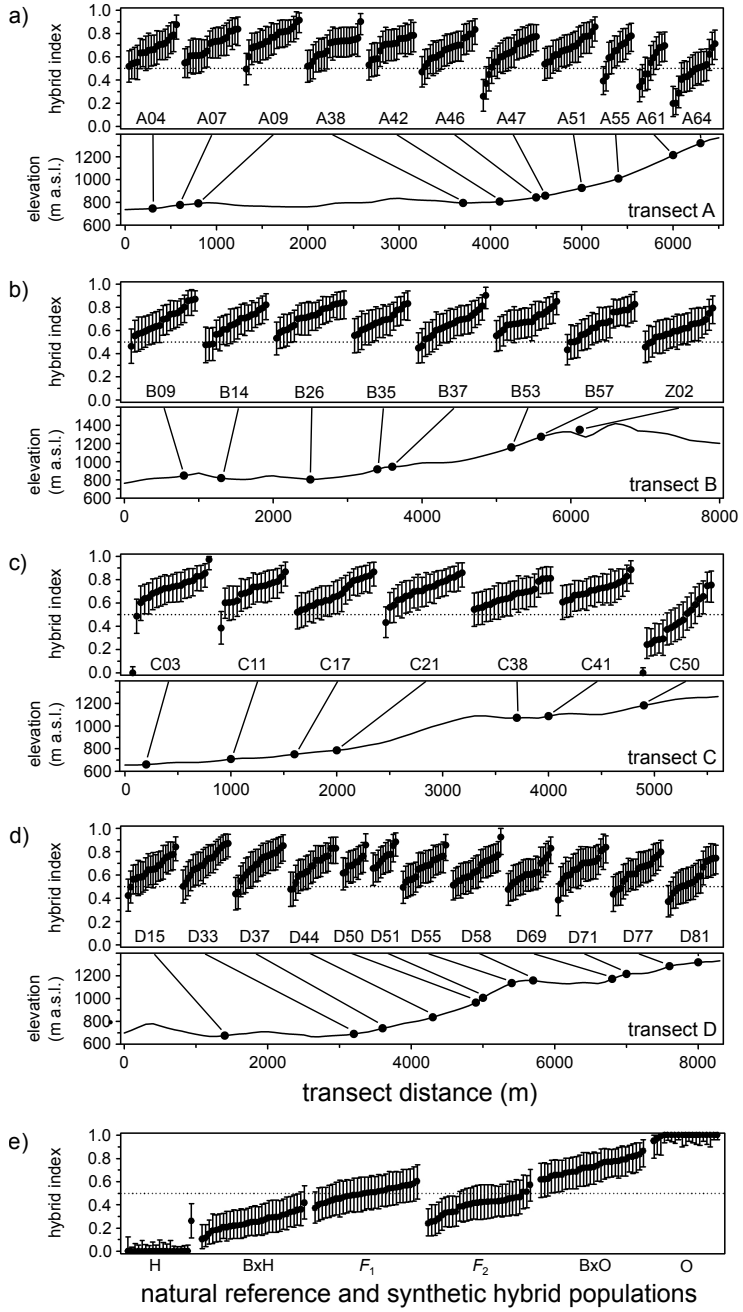

Figure 2.2: Hybrid indices (upper panel) and elevational profiles (lower panel) for the four investigated transects (a) Plattenhauser, (b) Rachel, (c) Scheuereck, (d) Lackenberg, together with (e) hybrid indices for the two reference populations (H: *S. hercynicus*, O: *S. ovatus*) and the synthetic hybrid classes (BxH: backcross to *S. hercynicus*, F_1, F_2, BxO: backcross to *S.ovatus*).

(100-420 bp and 150-420 bp) for band scoring. The optimal parameter settings were found at a minimum profiling of 1.5%, a minimum area of 0.3%, a shoulder sensibility of 0, a position tolerance of 0.13% and a fragment range of 100-420 bp. With these optimised parameters for the automatic analysis, the total dataset was scored into a 0/1-matrix, upon which the downstream statistical analyses were based.

2.3.3 Environmental variables

Fourty-eight environmental variables related to topography, geography, temperature, and precipitation were available, which were gained for all sampled reference points of the transects through the BIOKLIM project (Bässler et al., 2009). To handle potential correlation among the environmental variables, we selected non-redundant and biologically meaningful variables following an approach by Manel et al. (2010). For this purpose, we applied a principal component analysis (PCO) to all environmental variables. We then built groups of variables according to their correlation with the principal component axes and choose one to two variables per axis with the highest loadings. When choosing two variables, we took care that they were not correlated with each other at $r > | 0.8 |$. As a consequence, the following five climatic variables were included: mean temperature from May through August (°C, T_{0508}), minimum of the mean minimal monthly temperature (°C, T_{minmin}), monthly sum of precipitation in the month with lowest precipitation (mm, P_{monthmin}), difference in precipitation of month with highest minus month with lowest precipitation (mm, P_{diff}) and mean annual relative humidity (%, RH_{year}) (Table 2.1).

2.3.4 Analysis of population structure and introgression patterns

We analysed the binary matrix from AFLP fingerprinting by a principal co-ordinates analysis (PCoA) based on pair-wise Sørensen distances among the 727 operational taxonomic units (OTUs) using the program R v3.1.0 (R Development Core Team, 2012). We further used a Bayesian clustering approach as implemented in the program Structure v2.3.4 (Falush et al., 2007; Pritchard et al., 2010; Pritchard et al., 2000). The optimal number of genetic clusters *(k)* was calculated according to the method of Evanno et al. (2005) using k values ranging from 1 to 10 with five repetitions for each k. A dataset allowing for tetraploidy and recessive alleles was run using the admixture model with 50,000 generations including 15,000 burn-in generations. Genetic differentiation among populations was calculated as ϕ_{PT}, an analogous estimate

Table 2.1: Site-specific information for the investigated populations (Pop) along the four transects A-D of the Bavarian Forest National Park and for the reference populations of *S. hercynicus* (H) and *S. ovatus* (O). Number of individuals *(N)*, geographical location (latitude, longitude), elevation (elev), mean temperature from May through August (T_{0508}), minimum of the mean minimal monthly temperature (T_{minmin}), monthly sum of precipitation in the month with lowest precipitation (P_{monthmin}), difference in precipitation of month with highest-month with lowest precipitation (P_{diff}), and mean annual relative humidity (RH_{year}).

Pop	N	latitude	longitude	elev m a.s.l.	T_{0508} °C	T_{minmin} °C	P_{monthmin} mm	P_{diff} mm	RH_{year}
A04	18	48.918210	13.400899	746	13.28	-12.37	71.41	74.44	0.80
A07	20	48.920426	13.403234	777	13.25	-11.77	74.80	79.53	0.79
A09	20	48.921902	13.404791	792	13.19	-11.70	76.04	80.77	0.79
A38	20	48.943312	13.427378	795	13.81	-9.82	82.35	90.01	0.76
A42	17	48.946265	13.430494	807	13.48	-10.35	83.81	92.50	0.77
A46	20	48.949218	13.433612	843	13.35	-10.39	85.90	94.27	0.77
A47	20	48.949956	13.434391	859	13.31	-10.43	88.21	98.48	0.77
A51	19	48.952909	13.437509	926	13.06	-10.64	91.17	100.99	0.77
A55	11	48.955861	13.440627	1010	12.69	-10.96	96.12	106.99	0.76
A61	10	48.960289	13.445305	1215	11.87	-11.73	104.82	114.98	0.74
A64	16	48.962504	13.447644	1320	11.49	-12.12	108.21	115.59	0.72
B09	19	48.928608	13.366718	847	12.58	-13.09	77.23	72.54	0.81
B14	18	48.932900	13.368744	820	12.73	-12.90	75.83	74.07	0.80
B26	20	48.943203	13.373607	804	12.76	-12.97	76.33	79.55	0.80
B35	16	48.950930	13.377256	916	12.47	-12.92	79.10	77.37	0.78
B37	20	48.952647	13.378067	944	12.41	-12.91	79.59	77.44	0.78
B53	18	48.966383	13.384557	1157	12.29	-11.79	97.86	112.73	0.80
B57	20	48.969817	13.386180	1274	12.00	-11.74	104.08	123.32	0.80
Z02	20	48.974651	13.386520	1350	11.97	-11.97	102.79	117.47	0.77
C03	20	49.056601	13.290008	660	13.31	-10.04	89.16	98.49	0.83
C11	17	49.063221	13.294290	708	13.12	-10.16	86.15	95.98	0.83
C17	20	49.068185	13.297502	750	12.92	-10.33	87.43	96.17	0.83
C21	20	49.071495	13.299644	786	12.79	-10.46	90.38	98.75	0.83
C38	20	49.085560	13.308749	1073	11.35	-11.91	97.09	89.46	0.79
C41	18	49.088042	13.310357	1087	11.21	-12.00	95.69	85.59	0.79
C50	18	49.095488	13.315180	1182	11.07	-12.19	96.95	77.43	0.79
D15	20	49.097907	13.218356	675	13.05	-9.90	87.21	97.45	0.84
D33	19	49.099625	13.242863	690	12.97	-10.01	80.83	95.36	0.84
D37	20	49.100006	13.248309	740	12.83	-10.19	83.00	96.23	0.84
D44	19	49.100673	13.257840	836	12.38	-10.63	89.76	96.46	0.84
D50	10	49.101244	13.266009	965	11.84	-11.14	95.59	97.45	0.83
D51	10	49.101339	13.267371	1007	11.75	-11.28	98.54	99.62	0.83
D55	18	49.101719	13.272817	1136	11.62	-11.76	109.60	110.81	0.82
D58	20	49.102004	13.276902	1159	11.42	-11.75	110.26	107.96	0.82
D69	18	49.103047	13.291881	1173	11.03	-11.85	97.55	74.96	0.82
D71	20	49.103236	13.294604	1216	10.84	-12.00	96.88	70.55	0.82
D77	20	49.103804	13.302775	1284	10.65	-12.21	99.19	72.85	0.81
D81	20	49.104183	13.308222	1318	10.46	-12.36	98.17	67.93	0.81
H01	19	47.699840	10.183932	1010	na	na	na	na	na
O02	19	49.049740	12.257728	365	na	na	na	na	na

na - not available

of Wright's F_{ST} statistic for binary data using GenAlEx v6.5 (Peakall and Smouse, 2012).

Further, for each individual a maximum-likelihood based hybrid index (HI) value as implemented in the R package 'introgress' (Gompert and Buerkle, 2010) was calculated, which indicates the proportion of a certain parental ancestry. We used the reference populations (H, O) for calibration and further simulated 30 indidviduals of several hybrid classes (F_1, F_2, backcross to either of the pure species) using the R package 'AFLPsim' (Balao and García-Castaño, 2014) to check and compare the status of hybrids along the four transects. Additionally, 'introgress' features a multinomial regression-based method to estimate genomic clines and to quantify introgression for individual loci relative to the genomic background (Gompert and Buerkle, 2009). To test whether loci differ from a model of neutral introgression, we used the parametric approach as suggested by Gompert and Buerkle (2010), which has to be preferred over the permutation procedure, when parental populations do not exhibit fixed allele differences at all investigated loci. *P*-values were adjusted according to the false discovery rate (FDR) method (Benjamini and Hochberg, 1995). Only loci with allele frequency differentials $\delta > 0.25$ were analysed. Individuals from the reference populations were not included in the analysis to exclude the influence of a phylogeographic signal.

2.3.5 Search for taxon-specific AFLP bands

The identification of taxon-specific AFLP bands was carried out doing locus-wise chi-square tests for heterogeneity in contingency tables with the frequency of presence/absence of the band in OTUs from the reference populations and two OTUs from the populations under study as being characterised as most *S. hercynicus*/*S. ovatus*-like by the hybrid index. This approach may lead to an over-estimation of taxon-specific markers because not only bands private to one of the two taxa are found but also markers that show significantly different allele frequencies in the two taxa are considered taxon-specific. However, with respect to the general goal of the present analysis (i.e., testing the null hypothesis that there are taxon-specific loci that are under selection along the elevational transect) exaggeration of the number of taxon-specific loci is a more conservative approach because it minimises the chance of omitting possible taxon-specific loci (due to false-negative results of the chi-square tests) from comparison with results addressing their adaptive behaviour. Additionally, a modified dataset including only taxon-specific markers was analysed by PCoA and by the clustering approach of Structure. We used the same settings as above, but for Structure we reduced the values for k to 1-6.

2.3.6 Search for loci under selection

We have used three different approaches to search for AFLP markers that are either under direct selection themselves or closely linked to loci under selection. The intention was to find out (a) whether these selected loci show any correlation with climatological variables along all transects arguing for a climate-mediated selection regime in this hybrid zone and (b) whether selected loci coincide with loci that are considered taxon-specific. Since the first two frequentist F_{ST} outlier detection methods (i.e., MCHEZA, BayeScan) make the assumption of populations being in Hardy-Weinberg equilibrium and we were unaware about the presence of disomic inheritance of the tetraploids under survey, we applied a third spatial analysis method (Samβada) that does not use a population genetic approach.

(1) The program MCHEZA (Antao and Beaumont, 2011) is based upon the DFDIST kernel which searches for loci under selection based on the principle that genetic differentiation among populations is expected to be higher for loci under divergent selection than for the rest of the genome (Beaumont and Balding, 2004; Beaumont and Nichols, 1996). The program calculates locus-wise allele frequencies and F_{ST} values using the Bayesian method of Zhivotovsky (1999) and uses computer simulations to produce F_{ST} values for modelled AFLP loci under neutral conditions. As suggested by the software developers, we used the 'neutral mean F_{ST}' and the 'force mean F_{ST}' options to calculate a 'trimmed mean F_{ST}', which is then used as an estimate of the average 'neutral' F_{ST} uninfluenced by outlier loci. Loci with a noticeable high F_{ST} value were considered as being under divergent selection. The analysis was performed using 1,000,000 simulations, a 0.95 confidence interval (CI), and a FDR of 0.05 to correct for multiple testing. For all other parameters we used the default values.

(2) The program BayeScan v2.1 (Foll and Gaggiotti, 2008) was used to check for the reliability of the outlier loci found with MCHEZA owing to the fact that overall F_{ST} values calculated by MCHEZA may indicate exaggerated population differentiation caused by a single or a few populations with extreme allele frequencies. BayeScan uses a regression approach and a hierarchical Bayesian procedure to simultaneously estimate F_{ST} values for every locus in each population. For this analysis, we started with 50 pilot runs of 5,000 iterations each and finally ran a total number of 250,000 iterations with a burn-in of 50,000 and a thinning interval of 20, resulting in a sample size of 10,000 iterations. All other parameters were set to default. As above, we used a FDR of 0.05 for outlier detection.

(3) A third method to detect adaptive loci is the spatial analysis method as implemented in the program Samβada (Joost et al., 2007; Stucki et al., 2014).

In contrast to the two above mentioned procedures, it does not analyse AFLP data in a population genetic framework but is based on logistic regression models testing the strength of relationships between the presence or absence of an allele in a single individual genotype and environmental variables. As explained by Joost et al. (2008), this procedure has some advantages over the population genetic approaches by not being dependent on any assumption of inbreeding coefficients, therefore being applicable to sampling designs with only single individuals per location, and immediately returning direct relationships between environmental variables and candidate loci under selection coupled with these variables. Two statistical tests (a log-likelihood ratio test and the Wald test; Wald, 1943) are implemented to evaluate the significance of the correlation between presence/absence of an AFLP band at a locus and an environmental variable. We ran univariate models including the five selected climatic variables and used a significance threshold of 0.05, which was Bonferroni corrected for multiple comparisons.

To prove whether a climate-mediated selection regime is acting along the transect in the surveyed hybrid zone on a population level, we further investigated loci that passed the Samβada analysis and at least one of the F_{ST}-based methods described above for being adaptive with high probability. On the one hand we tested correlations between climatic variables detected by Samβada and frequencies of the present allele (band presence) by Spearman's rank correlation tests. On the other hand we used a partial constrained analysis of principal coordinates (partial CAP) to control for the effect of possible spatial structures (R package 'vegan'; Oksanen et al., 2014). The model consisted of a response data set (genetic differentiation) and two explanatory datasets (climatic variables and spatial structure). Genetic differentiation of populations was calculated as pairwise ϕ_{PT} values for all outlier loci in total by GenAlEx. The spatial structures were expressed as principal coordinates of neighbour matrices (PCNM) variables (R package 'PCNM'; Legendre et al., 2013). Only variables showing positive spatial correlation were used. In a first step, explanatory variables were forward-selected independently in each subset, and then the model was fit with the climatic variables retained controlling for spatial structures.

2.4 Results

2.4.1 AFLP fingerprinting results

AFLP fingerprinting of 790 OTUs including 19 OTUs of each pure reference population and 63 replicates for three selective primer combinations and an op-

timised automated band-scoring (Holland et al., 2008) yielded 681 polymorphic loci (D2: 227, D3: 219, D4: 235). An average Euclidian error rate of 12.4% was estimated among all replicates, being a reasonable rate for an automated band-scoring procedure (i.e., 6-13% or 9-18% given in Holland et al., 2008).

2.4.2 Population structure and introgression patterns

The overall population structures were weak. Neither PCoA (Figure 2.3) nor Structure could find clearly separated populations. In the case of Structure the estimated optimal number of clusters was $k = 1$. This result is also seen in a very low overall genetic differentiation among the populations of all four transects ($\phi_{\mathrm{PT}} = 0.035$, $P < 0.001$) ranging from population pairwise $\phi_{\mathrm{PT}} = 0.000$ (D50 vs. D51) to $\phi_{\mathrm{PT}} = 0.119$ (A64 vs. C03). The genetic differentiation between the reference populations was distinctly higher ($\phi_{\mathrm{PT}} = 0.221$).

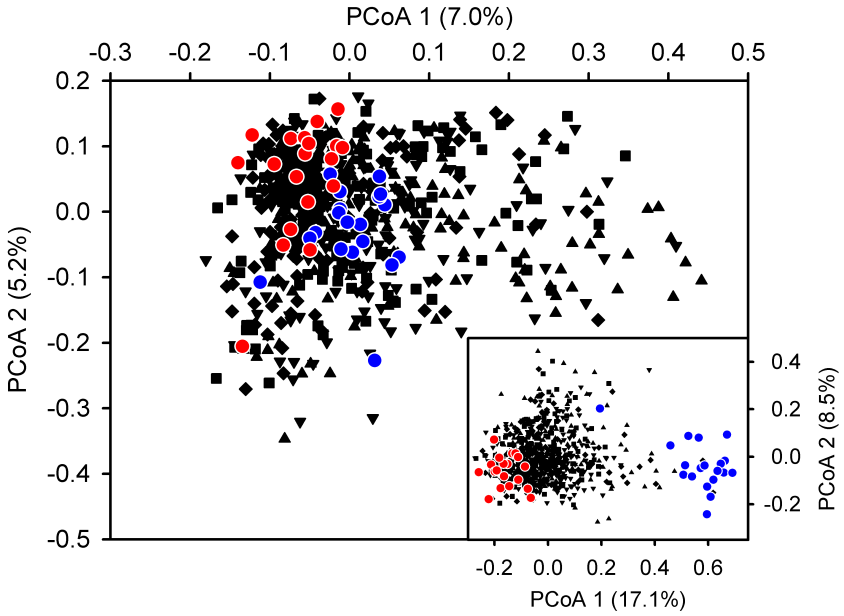

Figure 2.3: Principal co-ordinates analysis on the basis of pair-wise Sørensen distances for 681 loci and a subset of 62 taxon-specific loci (inset) resulting from AFLP fingerprinting. The proportions of explained variation are given on the axes.

The hybrid index shows a clear displacement of the hybrid swarm along the four transects in direction towards *S. ovatus* (Figure 2.2). According to the synthetic reference individuals, 72% (498) of all OTUs are assigned to the class of *S. ovatus* backcross individuals, 25% (170) belong to the F_1/F_2 classes, which cannot be distinguished from each other by the hybrid index. Only 2% (17) of all OTUs could be assigned to *S. hercynicus* backcross individuals. The

proportion of pure parental species was vanishingly small with *S. hercynicus* (HI < 0.05) represented by two and *S. ovatus* (HI > 0.95) represented by just one individual. Populations A64 and C50, both at very high elevations, were found to show the highest *S. hercynicus* ancestry.

On the basis of allele frequency differentials, we selected 59 loci having $\delta > 0.25$ for genomic clines analysis. Fourty-seven (80%) of these loci clearly deviated from a model of neutral introgression (Table 2.2, supplementary Table E.1, included CD), which means that the occurrence of a certain allele (band presence/absence) cannot be predicted by the hybrid status of a particular individual. Of those 47 loci, 26 loci showed a deficit (e.g., L100, L290 in Figure 2.4a) and 20 loci showed an excess (e.g., L297, L511 in Figure 2.4a) ancestry of *S. hercynicus* alleles in the *S. ovatus* genetic background. One locus (L193) showed an epistatic pattern, where each homozygous genotype is favoured in its native genomic background (supplementary Figure A.1, appendix).

The locus-wise chi-square tests identified 62 loci as taxon-specific from which 26 were private and one locus (L297) was fixed in both species (*S. hercynicus* – band present, *S. ovatus* – band absent). They correspond to 76% to the loci with the greatest information content, that were used in the genomic clines analysis. The results for the PCoA of the taxon-specific loci are represented in Figure 2.3 (inset). The scatter plot is similar to the results of the hybrid index with a high density of individuals near the *S. ovatus* cluster. The few individuals clustering with or near the *S. hercynicus* cluster are the same showing a low hybrid index, too. The optimal number of clusters for the Structure analysis was $k = 1$, again.

2.4.3 Outlier loci

The three methods used differed in the number of detected outlier loci (Table 2.2, also supplementary Table E.1, included CD for complete list). Thirteen loci were detected by MCHEZA. The forced and trimmed mean F_{ST} value, which served as basis of computation was 0.024, and the loci being under positive selection had significantly higher values in a range between $F_{\mathrm{ST}} = 0.065$ and $F_{\mathrm{ST}} = 0.188$. Twice as many loci (31) as detected by MCHEZA, were detected by BayeScan. Of these, 19 loci were detected exclusively by BayeScan and eight loci were additionally detected by MCHEZA. This shows that the characterisation of loci as outlier loci is not independent of the inference method and the underlying model.

The logistic regression model testing implemented in Samβada resulted in significant correlations between marker frequencies at 14 loci and the given five climatic candidate variables. Nine of these loci coincided with loci found by MCHEZA and ten of these loci coincided with loci found by BayeScan. In

Table 2.2: Characterisation of 15 outlier loci found as being under selection by at least two of the three methods implemented in MCHEZA, BayeScan, and Samβada. For each locus, estimates of F_{ST} and associated significance values obtained using MCHEZA and BayeScan are given. Outlier loci found by Samβada are documented together with the environmental variable(s) showing significant correlations with the presence of the dominant allele ordered according to descending Wald scores. Additionally, Fisher's exact test statistics (X^2, P_{adj}) are given, indicating whether a locus was found to be taxon-specific. The last column shows the allele frequency differentials (δ) along with estimates of the genomic clines analysis as calculated by Introgress. 'Genomic cline' indicates whether a locus shows a significant excess (H+) or deficit (H-) of *S.hercynicus* genotypes in a *S. ovatus* background. All P-values (P_{adj}) were adjusted according to Benjamini and Hochberg (1995) to account for multiple testing. Significant values are in bold. See also supplementary Table E.1, included CD for a complete list.

Locus	**MCHEZA**		**BayeScan**		**Samβada**	**taxon-specificity**		**Introgress**			
	$\boldsymbol{F_{ST}}$	$\boldsymbol{P_{adj}}$	$\boldsymbol{F_{ST}}$	***q*-value**		X^2	$\boldsymbol{P_{adj}}$	δ	**lnL ratio**	$\boldsymbol{P_{adj}}$	**genomic cline**
L088	**0.086**	**<0.050**	**0.081**	**<0.001**		0.171	>0.999	0.00	na	na	na
L100	**0.096**	**<0.050**	**0.085**	**<0.001**	$P_{monthmin}$, RH_{year}, T_{0508}	**25.846**	**<0.001**	0.49	**34.166**	**0.010**	**H-**
L185	0.047	>0.050	**0.059**	**0.010**	T_{0508}	**34.381**	**<0.001**	0.74	**81.920**	**<0.001**	**H+**
L186	**0.069**	**<0.050**	0.017	0.789	RH_{year}	na	na	0.00	na	na	na
L203	**0.083**	**<0.050**	**0.070**	**0.001**	P_{diff}, $P_{monthmin}$	**11.455**	**0.010**	0.24	na	na	na
L209	**0.096**	**<0.050**	0.017	0.772	P_{diff}	na	na	0.00	na	na	na
L290	**0.076**	**<0.050**	**0.080**	**<0.001**	$P_{monthmin}$, RH_{year}	**11.629**	**0.017**	0.40	**39.346**	**0.005**	**H-**
L294	0.063	>0.050	**0.065**	**0.002**	RH_{year}	0.000	>0.999	0.00	na	na	na
L297	**0.123**	**<0.050**	**0.123**	**<0.001**	$P_{monthmin}$, T_{0508}, RH_{year}	**42.000**	**<0.001**	1.00	**317.974**	**<0.001**	**H+**
L337	**0.075**	**<0.050**	0.036	0.097	$P_{monthmin}$, T_{0508}	1.024	>0.999	0.03	na	na	na
L358	**0.188**	**<0.050**	**0.159**	**<0.001**	RH_{year}, T_{minmin}	0.359	>0.999	0.03	na	na	na
L359	0.060	>0.050	**0.073**	**0.001**	$P_{monthmin}$, T_{0508}	**11.958**	**0.010**	0.43	**257.897**	**<0.001**	**H+**
L502	0.048	>0.050	**0.068**	**0.001**	T_{0508}, T_{minmin}	**21.000**	**<0.001**	0.39	**209.072**	**<0.001**	**H+**
L511	**0.068**	**<0.050**	**0.058**	**0.006**	T_{0508}, $P_{monthmin}$	**19.056**	**0.001**	0.56	**98.497**	**<0.001**	**H+**
L565	**0.065**	**<0.050**	**0.071**	**<0.001**		3.231	0.661	0.05	na	na	na

na - not available

summary, 15 loci were detected by at least two of the methods applied (Table 2.2), of which six loci were detected by all three methods. Of the 15 outlier loci jointly detected by MCHEZA and BayeScan, eight loci were additionally detected as being taxon-specific. Except locus L203 all of these eight loci showed a departure from a model of neutral introgression with five loci having an excess and two loci having a deficit of the *S. hercynicus* genotype in the *S. ovatus* background.

2.4.4 Correlation of outlier loci and climatic variables

All of the five given climatic variables showed a significant relationship to at least one locus in the Samβada analysis. For seven loci, and therefore most frequently, correlations occurred with P_{monthmin}, RH_{year}, and T_{0508}. P_{diff} and T_{minmin} showed significant correlations with merely two loci, respectively.

Loci significantly correlated with at least one of the given climatic variables found by the logistic regression model (Samβada) were further tested for correlation with climate on a population basis. Except two correlations, all tested loci were confirmed by Spearman's rank tests being significantly ($P < 0.05$) correlated with the given climatic variables. Spearman's rank correlation coefficient values ranged from $r_{\text{S}} = \mid 0.337 \mid$ to $r_{\text{S}} = \mid 0.756 \mid$ (supplementary Table E.1, included CD). Figure 2.4 exemplifies four loci and their correlation between climate and band frequency.

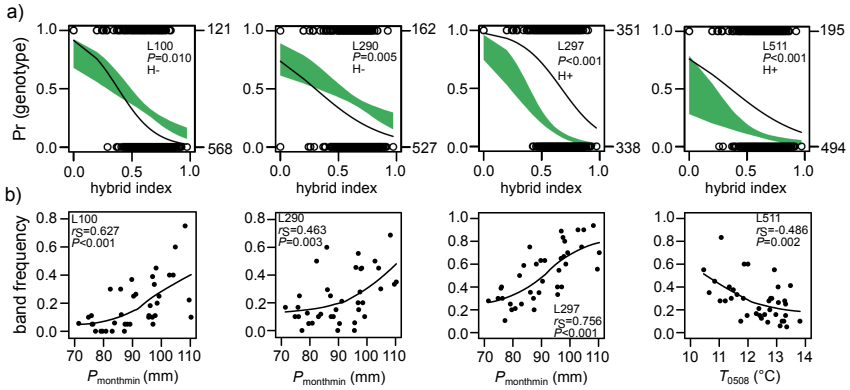

Figure 2.4: Fitted genomic clines for a subset of loci and their relationships between climate and population-wise band frequencies. (a) Genomic clines of two loci showing a deficit (L100, L290) and two loci showing an excess (L297, L511) ancestry of *S. hercynicus* alleles in the *S. ovatus* genetic background. Solid line: probability of *S. hercynicus* genotype as function of hybrid index; green area: neutral model; circles: individuals with *S. hercynicus* genotype on top line and individuals with *S. ovatus* genotype on bottom line with quantity given on the right axis. (b) Correlation of climate and population-wise band frequencies. Spearman's rank statistic is given in the plot, respectively. Solid line: loess curve.

To check the influence of spatial structures we run a partial CAP. The most parsimonius model after forward-selection contained P_{monthmin}, RH_{year}, and T_{0508} as explanatory climatic variables conditioned on the first PCNM variable. About 28% of the total variance were explained by the climatic variables and 7% were explained by the spatial structure. Running an ANOVA-like permutation test, only P_{monthmin} (*pseudo-F* = 2.973, $P = 0.004$) and T_{0508} (*pseudo-F* = 2.630, $P = 0.013$) showed a significant effect, while the effect of RH_{year} was not significant (*pseudo-F* = 1.704, $P = 0.083$). There is a strong correlation between RH_{year} and latitude (r_{S} = -0.933, $P < 0.001$) that is represented by the first PCNM variable. Therefore, one cannot assume that the genetic differentiation among populations of the transects is a consequence of RH_{year}, but also is caused by the spatial structure.

2.5 Discussion

As already mentioned in the introduction, the present study is a continuation of Oberprieler et al. (2015), who investigated hybridisation and climatic selection along the 'Plattenhauser' transect (A). In their preliminary study, Oberprieler et al. (2015) detected highly intensive introgression along the whole transect, with a small number of outlier loci showing significant correlations with climatological variables, but these outlier loci were not taxon-specific and it was assumed that there is no climate-mediated selection, which could maintain the two species (Oberprieler et al., 2015). In most instances, we here confirm the preliminary results of Oberprieler et al. (2015), but we also shed light on new aspects concerning introgression.

Addressing the first question about the extent of hybridisation between *S. hercynicus* and *S. ovatus* along the four transects, we can reconfirm the finding of Oberprieler et al. (2015). In contrast to these preliminary results, however we here did not find the populations along the transects as being hybrid swarms with different admixture proportions of both parental genotypes, but we identified them as being backcross individuals towards *S. ovatus* to a very high proportion, explaining the overall low genetic differentiation among populations. This discrepancy can be attributed to the origin of reference populations. While Oberprieler et al. (2015) used the most extreme genotypes from a former study in the Bohemian Forest (Oberprieler et al., 2010), we here selected our reference populations according to the nearest known pure stands of either species. We assume that the reference individuals used in the study by Oberprieler et al. (2015), do not represent pure parental genotypes leading to a bias in population genetic structures. On the other hand, one has to keep in mind that since the reference population of *S. hercynicus* in the

present study was not found in a region closer than 250 km, and might have a long, independent history of diversification what in turn could lead to a bias in population structures, too.

Studies in Louisiana Irises, *Morus*, *Orchis*, *Helianthus* or *Rhododendron* (Arnold et al., 2010; Burgess et al., 2005; Jacquemyn et al., 2012; Scascitelli et al., 2010; Tagane et al., 2008) show that asymmetric introgression, is a common phenomenon in plants. There are several causes that might lead to this pattern, like differential abundance of parental taxa (Burgess et al., 2005) or differential gamete production, fertilization and offspring survival (Tiffin et al., 2001). The question which mechanisms act in the *S. hercynicus*-*S. ovatus* system, cannot be answered in the present study as we need to do further studies on this topic.

Interestingly, the genetic structure along the four transects clearly differs from the one found by Raudnitschka et al. (2007), who detected pure *S. hercynicus* stands on the summit plateau of Mt. Brocken in Harz Mountains (Anhalt-Saxony, Germany), pure *S. ovatus* stands at the lowermost elevations, and hybrid swarms at intermediate elevations. A similar pattern was found in another well-studied *Senecio* hybrid system on Mt. Etna, Sicily (Italy), where *S. aethnensis* grows on high elevations, *S. chrysanthemifolius* grows on low elevations and hybrid individuals of both species are formed at intermediate elevations (Brennan et al., 2009; James and Abbott, 2005). In our study, we only found two populations, i.e., the uppermost populations of transect A and C, which showed at least a weak relationship to *S. hercynicus*. This suggests that disruptive ecological selection along the transects is weak and cannot re-establish the biological species, as was already pointed out by Oberprieler et al. (2015).

Nevertheless, and thereby answering the second question, we could detect 13 outlier loci that were related to climatic variables concerning precipitation and/or temperature. In contrast to Oberprieler et al. (2015), who did not find taxon-specific outlier loci related to climatic variables, from the present study, more than half of the 13 outlier loci were taxon-specific. We assume that these loci are probably linked to genes that play a role in heat/cold tolerance or drought stress. Thus, precipitation and temperature could be drivers of population adaptive divergence along the transects. Several other studies have already identified precipitation and/or temperature as environmental factors driving plant and animal adaptation (Bothwell et al., 2013; Henry and Russello, 2013; Manel et al., 2010, 2012; Nunes et al., 2011; Poncet et al., 2010; Richardson et al., 2009). Actually, Zulliger et al. (2013) could show that respective outlier loci can be transferred among closely related taxa corresponding to the idea of semipermeable species boundaries.

According to Rieseberg (2001) there is no complete barrier to gene flow

among closely related species with the exception of geographical isolation. It is rather the case that neutral or advantageous alleles move freely between hybridising species unless they are linked to regions contributing to reproductive isolation (Baack and Rieseberg, 2007). Thus, the future trajectory of hybridising species (fusion vs. speciation) depends on how strong genes are coadapted and how often these coadaptations are broken up (Wu, 2001). Addressing the third question, we here found 47 loci that deviated from a model of neutral introgression. Thereof, 20 loci showed an increased probability of *S. hercynicus* genotypes to introgress into the *S. ovatus* genetic background. Among these, five loci correlated with precipitation and/or temperature. We assume that this may lead to an adaptation of the *S. ovatus*-like genotypes to climatic conditions and therefore shorter vegetation periods in higher elevations. On the other hand, we also found 27 loci that showed a decreased probability of *S. hercynicus* genotypes to introgress into the *S. ovatus* genetic background. These loci may suggest candidate regions of the genome that are involved in reproductive isolation (Hamilton et al., 2013) and thus could be involved in species maintenance. However, the population genetic structures show a dominance of *S. ovatus*-like genotypes. Therefore, we assume the acting selection regimes to be very weak to maintain the two biological species. Further, climate change may enhance the genetic swamping of *S. hercynicus* by *S. ovatus* by causing an upwards shift in species distribution (Thuiller, 2007). Finally, our results exclude the formation of a stable hybrid zone either following the 'tension zone model' or the 'bounded hybrid superiority model'. It is rather a moving zone following the 'advancing wave model', but further studies are needed to confirm these findings.

Conclusion

Hybridisation of *S. hercynicus* and *S. ovatus* is in an advanced stadium and most of the individuals can be assigned to the *S. ovatus*-backcross class. Nevertheless, we found signs of ecological selection acting along the transects, but also signs of adaptive introgression. Up to now, we cannot make clear statements on fitness differences, for example resistance against herbivores, between the two species and probably there are other forces acting so that *S. ovatus* gains advantage over *S. hercynicus*. Further studies concerning herbivory (i.e., transplantation experiments and food choice experiments, see chapters 3 and 4) are on the way and should shed light on this context.

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CHAPTER 3

Ecological selection along an elevational gradient in three central European *Senecio* L. (Compositae, Senecioneae) species and their hybrids

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3.1 Abstract

Elevational gradients with adjacent distribution zones of closely related and hybridising plant species provide a suitable natural set-up for studying evolutionary processes like adaptation and speciation. Transplantation experiments along these gradients are a straightforward strategy to infer fitness differences among genotypes and to pinpoint intrinsic and extrinsic factors that govern elevational stratification. *Senecio germanicus, S. ovatus,* and *S. hercynicus* are vertically vicariant species of the *S. nemorensis* syngameon that show mutual hybridisation in their range overlaps. We here report on the results of transplantation experiments with the three species and their reciprocal F_1 hybrids along an elevational transect in the Bavarian Forest, SE Germany, in which we measured leaf area loss through mollusc herbivores and through withering caused by climatic influences. We find a strong correlation of the amount of the remaining photosynthetically active leaf area and rhizome dry weight as a proxy for overall viability of plants and their reproductive and propagational success. In the *S. hercynicus-S. ovatus* system, higher damage values caused by herbivores in the former species and by withering in the latter one, along with intermediate values found for hybrid genotypes, suggests a trajectory following an 'advancing wave model' of introgressive hybridisation. In the *S. germanicus-S. ovatus* system, the more effective phytochemical herbivore deterrent strategy of *S. ovatus,* together with its higher susceptibility to colder conditions should follow a 'mosaic hybrid zone model' and eventually to a patchy distribution of pure parental and hybrid populations governed by the heterogeneity of climatic conditions at lower elevations.

3.2 Introduction

Elevational gradients with their steep changes in abiotic (temperature, moisture, hours of sunshine, UV radiation, season length, geology; Körner, 2007) and biotic features (pollinator and herbivore communities and densities) provide perfect natural laboratories to study plant adaptation and its consequences for the evolution of plant distributional ranges. A number of studies aiming at the understanding of determinants shaping plant distributions have used elevational gradients to study the role of features of the physical and biological environment in this pattern formation (e.g., Bruelheide and Scheidel, 1999, 2001, 2004, 2005; Scheidel et al., 2003). From an evolutionary point-of-view, situations in which closely related species inhabit adjacent elevational zones are most informative for pinpointing ecological factors that may have played decisive factors during the divergence of taxa ('ecological speciation'; Schluter, 2000; Levin, 2004), as exemplified in the island models of *Cyanea* on the Hawaiian (Givinish et al., 1995) and *Argyranthemum* on the Macaronesian Archipelago (Francisco-Ortega et al.,1997). If adjacent species ranges overlap and hybridisation between the closely related taxa occurs, the set-up is even more instructive because the formation, shape, and fate of the resulting hybrid zone – with its more or less stable balance between natural selection on the one and gene flow on the other hand – may allow insights into the genetic and ecological basis of species involved (Rieseberg et al., 1999; Coyne, Orr, et al., 2004; Orr, 2005; Moccia et al., 2007).

The structure and fate of hybrid zones may broadly vary depending on the fitness of hybrid genotypes in relation to their parental ones (Fritz, 2001; Abbott and Brennan, 2014). If hybrids have lower fitness than the two parental taxa, the 'tension zone model' predicts that hybrids should be in general restricted to a narrow zone at the clinal transition between the two hybridising species (Barton and Hewitt, 1985). In the case of altitudinal gradients, the hybrid zone should be limited to a narrow belt at intermediate elevations, where hybrids are continuously formed by gene flow across the boundary between distribution ranges, but selected against in an environmentally independent (intrinsically determined) manner (Abbott and Brennan, 2014). The 'bounded hybrid superiority model', on the other hand, describes situations, in which hybrids have higher fitness than either parental species in transition zones, but lower fitness in parental habitats (Moore, 1977). In this situation, selection against hybrids is environment-dependent (extrinsic selection) and hybrid zones tend to occupy ecotones; in the case of altitudinal belts, again at intermediate elevations (Abbott and Brennan, 2014). In contrast to these two models predicting more or less stable patterns to emerge, the 'advancing wave model' describes the dynamic change of patterns that result from situations, where

hybrids are fitter in one of the two parental ranges but lower in the other (Fritz, 2001). Here, in an again environmentally determined selection regime, the hybrid zone should shift until a balance between selection and gene flow (as described in the 'bounded hybrid superiority model') is reached or the less fit parent is replaced completely by hybrid genotypes and subsequently by the other parental species ('extinction by hybridisation' or 'genetic swamping'; Ellstrand and Elam, 1993; Ellstrand et al., 1999; Levin et al., 1996; Wolf et al., 2001).

Damage by herbivory is a very important component of environment-dependent, extrinsically mediated fitness of a plant individual (Massad, 2013) and has been shown to considerably influence plant abundance and distribution (Maron and Crone, 2006). For example, Bruelheide and Scheidel (1999) could demonstrate that the lower elevational boundary of the distribution range of *Arnica montana* is determined by herbivory through slugs and snails. In hybrid systems, differences among genotypes influencing their resistance to herbivores and pathogens may proximately determine their relative fitness (Fritz, 2001) and thus ultimately designate the evolutionary trajectory of a single hybrid swarm population or a whole hybrid zone. According to Fritz (2001), four main patterns are conceivable: Hybrids can be more susceptible (susceptibility pattern) or more resistant (resistance pattern) than their parental species. They can also show intermediate resistance relative to their parental taxa (additive pattern) or may approach one of the two parents in this respect (dominance pattern). As demonstrated by Fritz et al. (1994, 1996) in the *Salix eriocephala-S. sericea* hybrid system or Dobson et al. (2011) in the Louisiana *Iris* system, herbivore taxonomy and temporal dynamics of herbivore communities along with the formation of backcrossed hybrid genotypes may further add to the complexity of situations and, in the latter case, may even lead to the transfer of herbivore resistance from one parental species to the other (e.g., in *Helianthus*; Whitney et al., 2006).

The three Central European representatives of the *Senecio nemorensis* syngameon exhibit a distinct distribution pattern showing vertical vicariance, with preferences of lower elevations and more continental climatic conditions by *S. germanicus* Wallr. subsp. *germanicus,* intermediate ones with more oceanic conditions by *S. ovatus* (P.Gaertn., Mey. & Scherb.) Willd. subsp. *ovatus,* and high elevations with a high montane or subalpine climate by *S. hercynicus* Herborg var. *hercynicus.* While some overlap of distribution ranges of pairs of species is observed, differences in flowering time quite often prevents hybridisation of the otherwise interfertile and artificially crossable species (Herborg, 1987). However, sporadic formation of hybrids with intermediate flowering time and unreduced intrinsic fitness values (i.e., viability, fertility) may lead to introgressive hybrid swarms with different frequencies and extents in different

parts of the distribution ranges of the parental species. Based on morphological, phytochemical (pyrrolizidine alkaloids), and genetic data, introgressive hybridisation could be detected both in the *S. hercynicus-S. ovatus* system in the Central European highlands (Harz National Park, Anhalt-Saxony, Germany, Raudnitschka et al., 2007; Bohemian Forest, Bavaria, Germany, Oberprieler et al., 2010) and in the *S. germanicus-S. ovatus* species pair (Naab valley, Bavaria, Germany, Oberprieler et al., 2011). Since F_1 hybrids and backcrossed individuals usually show no or only marginally reduced intrinsically mediated reduction in fitness parameters (i.e., pollen fertility, seed set, or germination rate) relative to the parental taxa concerned (Herborg, 1987; Oberprieler, 1989; Oberprieler et al., submitted), the question arose whether extrinsic fitness parameters in general and herbivory-mediated fitness reduction in particular may explain the elevational vicariance of the three species and determine the evolutionary fate of populations and species after occurrence of introgressive hybridisation.

To answer questions of herbivory-mediated fitness differences among the three *Senecio* species and between parental taxa and their F_1 hybrids along elevational gradients, we established experimental plots with the taxa/hybrids concerned along a transect in the Bavarian/Bohemian Forest range (Bavaria, Germany) at four elevations (300 m, 600 m, 900 m, and 1200 m) in two consecutive vegetation periods (2011, 2012). To assess taxon- and elevation-dependent reduction of photosynthetically active leaf area caused by herbivory and climatological factors, we monitored the transplantation experiment in intervals of two weeks and scored genotypes for loss of leaf area caused by leaf consumption or withering. In order to estimate the cumulative effects of leaf-area loss on the viability of these clonally propagating perennial geophytes, we additionally assessed rhizome weight by the end of the vegetation periods. Statistical analysis of these parameters should allow us to answer the following questions: (1) How is intensity of herbivory on leaves of members of the *Senecio nemorensis* syngameon related to elevation in general? (2) Are there significant differences in leaf-area loss among the three *Senecio* species under study that could explain their elevational vicariance? (3) Do herbivory-related differences exist between hybrid taxa and their parental species that would allow us inferring the selection regime and specifying of the hybrid model in action and, as a consequence, predicting the evolutionary trajectory naturally occurring hybrid swarms may take?

3.3 Material and methods

3.3.1 Study species

Senecio germanicus, S. hercynicus, and *S. ovatus* are tetraploid ($2n = 40$), insect-pollinated, perennial geophytes and reported to be self-incompatible (Herborg, 1987; pers. obs. 2011). They are capable of producing large numbers of wind-dispersed fruits (achenes) and also propagate vegetatively by formation of stolones. All three are members of plant communities typical of succession stages after natural or anthropogenic disturbances (e.g., forest clearings). Conspicuous differences in morphology between the study taxa concern the leaf bases of the upper cauline leaves, which are sessile in *S. germanicus* and *S. hercynicus* and petiolate in *S. ovatus.* Further, the dimension and shape of the capitula is discriminating the three taxa. *Senecio germanicus* and *S. hercynicus* are characterised by larger capitula with a higher number of tubular/disc florets and involucral bracts than *S. ovatus.* In addition, in *S. germanicus* the supplementary involucral bracts are furnished with long eglandular hairs and also the lower part of the stem possesses long eglandular hairs. *Senecio hercynicus* is characterised by the possession of glandular hairs on leaves, the upper part of the stem and on the supplementary bracts of the capitulum. In contrast to these two species, in *S. ovatus* only articulate hairs are found.

When growing in a common-garden experiment at the University of Regensburg, Bavaria (Germany), the three species exhibit considerable differences in phenology (pers. obs. 2011, 2013): although the shoots emerge nearly simultaneously around the middle of April, *S. hercynicus* grows much faster than the other two species and flowers about four weeks earlier than *S. ovatus,* while *S. ovatus* in turn flowers about two weeks earlier than *S. germanicus.* The average flowering time of *S. hercynicus* is in general between June and July, for *S. ovatus* between July and August and for *S. germanicus* between July and September. However, isolation by flowering time is not complete and hybridisation is possible, with corresponding F_1 hybrids showing intermediate flowering times (pers obs. 2013).

3.3.2 Plant cultivation and crossings

Parental individuals were collected before commencement of flowering and transplanted into pots in late spring and early summer 2010. We used plants from populations, for which the morphological and/or genetic constitution had been analysed before and hybridisation with one of the other species could be excluded. *Senecio germanicus* was collected near Penk-Distelhausen,

Bavaria, Germany (population 9 of Oberprieler et al., 2011), *S. hercynicus* from Eschacher Wald near Kempten, Bavaria, Germany (population 1 of Oberprieler, 1989, 1994) and *S. ovatus* near Unterlichtenwald, Bavaria, Germany. Seeds of pure species were collected in the same populations later in that year. Additionally, seeds of *S. ovatus* were collected in 2012 from a second population near Falkenstein, Bavaria, Germany, because seed production in the first population was too low.

Crosses were made in the botanical garden of the University of Regensburg. To prevent insect-pollination, inflorescences were covered with self-made bags from commercial pollen gauze. *Senecio ovatus* and *S. germanicus* were hand-pollinated between middle of July and beginning of August 2010. *Senecio ovatus* and *S. hercynicus* were hand-pollinated between end of June and beginning of July 2011. To increase yield of hybrid seed, previously harvested and frozen (-18°C) pollen of *S. hercynicus* was used to hand-pollinate *S. ovatus* for an extended period until middle of July 2011. Seeds were harvested in the beginning of September 2010 (*S. germanicus-S. ovatus* crosses) and in the middle of August 2011 (*S. hercynicus-S. ovatus* crosses) and stored at 5°C under dark and dry conditions. To adjust all individuals to a homogenous starting point in their development, the pericarp and testa of achenes were removed and the embryos were kept on moistened filter paper in Petri dishes under greenhouse conditions (20°C/12°C, 14 h day / 10 h night) for about one week. Seed preparation for the first experimental year (*S. hercynicus, S. ovatus, S. germanicus,* and the reciprocal crosses of the two latter species) started by the end of November 2010 and for the second experimental year (*S. ovatus, S. hercynicus,* and their reciprocal crosses) in the middle of January 2012. Seeds for preparation were chosen randomly. One week old plants were transplanted into seed trays in a 4:2:0.5 compost : Einheitserde® Classic (Sinntal – Altengronau, Germany) : sand mixture. After about seven weeks they were planted into 12-cm pots. Green house conditions and soil mixture were kept constant during growing. To control plant pests we used glue panels against sciarid flies and plant protection oil against aphids. Plants were transferred to experimental fields of the botanical garden for acclimatisation in the middle of March in both years.

3.3.3 Genotyping

To check whether the crosses were successful (i.e., hybrid genotypes were formed), we screened all seedlings for a sequence difference in the intergenic spacer one of the nuclear ribosomal repeat (nrDNA ITS1) when the primary leaf was completely developed. Without prior DNA extraction, a direct PCR amplification of this marker was carried out with primers P1: 5' – GGA

AGT AAA AGT CGT AAC AAG G – 3' (White et al., 1990) and P2: 5' – CTC GAT GGA ACA CGG GAT TCT GC – 3' (Ochsmann, 2000). PCR reactions were carried out in a total volume of 11 µl containing 6.25 µl of a twofold concentrated Master Mix RED with 1.5 mM $MgCl_2$ (Ampliqon, Odense, Denmark), 0.25 µl of each primer (10 pmol μl^{-1}, and a sample disc of the primary leaf punched with a 0.5 mm Harris Uni-Core^{TM} (FisherScientific, Schwerte, Germany). Amplification was carried out at 94 °C for 5 min and for 35 cycles at 94 °C for 1 min, at 52 °C for 1 min, at 72 °C for 1 min and a final extension at 72 °C for 5 min. The PCR product (length 410 bp) was digested with *Bse*GI (FisherScientific, Schwerte, Germany) resulting in a genotype-specific digestion with no digestion for genotype 1 *(S. ovatus)* and a digestion at position ITS1-90 resulting in two fragments with 280 and 130 bp length for genotype 2 *(S. germanicus, S. hercynicus).* F_1 hybrids were identified according to the mixture of both restriction patterns (three bands).

3.3.4 Transplantation experiment

To examine herbivory differences among the three pure species and their F_1 hybrids along an elevational transect, we set up three plots at each of four different elevations (300 m, 600 m, 900 m, 1200 m a.s.l.) ranging from Deggendorf, Germany to the mountain Großer Falkenstein in the Bavarian National Park, both Bavaria, Germany (Figure 3.1; supplementary Table A.1, appendix). We tried to keep shading of plots over all elevations as constant as possible. Plots at the same elevation were about 100 m apart from each

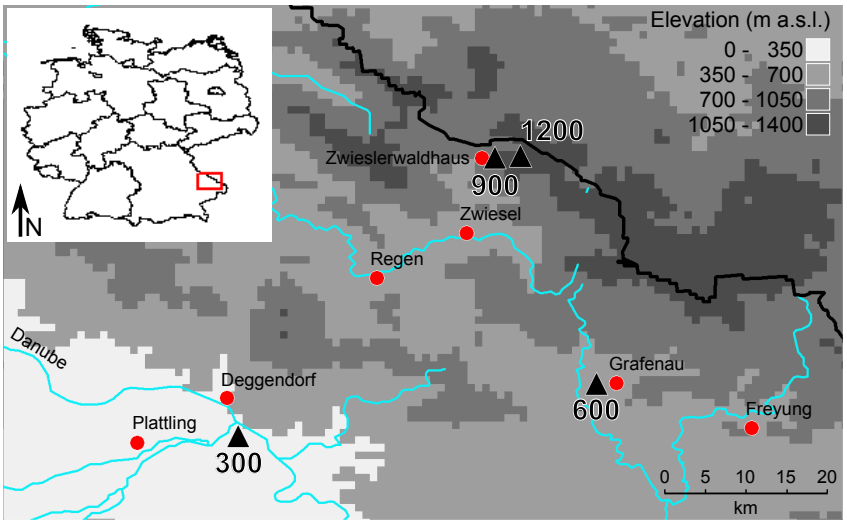

Figure 3.1: Geographical location of plots in eastern Bavaria, SE Germany.

other. Each plot was arranged in a randomised latin square design comprising 23 plants (five individuals of *S. hercynicus, S. ovatus, S. germanicus* and *S. ovatus* × *S. germanicus,* respectively and three individuals of *S. germanicus* × *S. ovatus*) in the year 2011 or 20 plants (five individuals of *S. hercynicus, S. ovatus, S. hercynicus* × *S. ovatus* and *S. ovatus* × *S. hercynicus,* respectively) in the year 2012. The distance between two neighbouring plants was about 80 cm. Additionally, five DIN A4 card boards were installed on the plots at random places under inclusion of free places in the latin square design to assess mollusc density. The plants were transplanted with their pots to the two lower elevations in the middle of April (13 April 2011, 17 April 2012) and to the two upper elevations four weeks later (09 May 2011, 14 May 2012). After disturbance of the plots at 600 metre by an unknown, larger animal in the year 2012, all plots were surrounded by dabbers from sheep's wool and dog's hair. Additionally, aluminium foil pennants and rags soaked with Certosan® (Flügel, Osterode, Germany), a biological browsing repellent, were arranged around the plots. Plots were re-visited every 14 days for scoring of herbivory intensity. To assess loss of leaf area, leaves of the main stem and of the paraclades (side branches) were classified into a maximum of five classes, respectively, according to their leaf dimension. For each class, the number of leaves were counted and one representative leaf per class was chosen for measurement of its width and length. Every leaf in the respective class was assigned to one of six damage classes (undamaged, up to 10%, 25%, 50%, 75%, or 100% loss of leaf area).

3.3.5 Assessment of leaf lamina loss

We determined the three leaf parameters of consumed leaf area *(cla),* photosynthetically active leaf area *(pala),* and brown leaf area *(bla)*, together with the rhizome dry weight *(rdw)* to draw conclusions about the relative herbivory resistance and its consequence for storage efficiency of the taxa under study. One week before transplanting plants to the field, ten individuals per taxon were chosen and all unfolded leaves, fixed in a picture frame with scale, were photographed (about 10 leaves per individual). Leaves were then measured for their width, length and area using ImageJ v1.44p (Wayne Rasband, downloadable at http://rsbweb.nih.gov/ij/) to calculate a taxon-specific coefficient k for leaf area determination (leaf area = leaf width × leaf length × k). Additionally, the total leaf area *(tla),* which is the total leaf area produced by the plant up to a specific date including consumed and withered leaf areas, was calculated by summing up all leaf areas of the maximally ten leaf classes. The consumed leaf area *cla* was then calculated by summing up the leaf areas times their mean of the respective damage class (0%, 5.5%, 18%, 38%, 63%, 88%) assessed in the field and was expressed as percentage of the total leaf area *tla.* The

photosynthetically active leaf area *(pala)* was calculated by summing up only the green leaf areas from which *cla* of the green leaves is subtracted. *pala* was expressed as percentage of *tla,* too. The brown leaf area *bla* represents leaf area withered by exogenous and/or endogenous factors, which was calculated by subtracting the percentages of *pala* and *cla* from 100%.

To assess rhizome dry weight *rdw,* all plants were removed from experimental plots in the beginning of October (06 October 2011, 01 October 2012) and brought to the laboratory. Rhizomes were washed and all green sprouts and roots were cut off. Subsequently, rhizomes were dried for 72 h at 65 °C and weighed.

3.3.6 Statistical data analysis

Statistical analyses were carried out with R v2.14.2 (R Development Core Team, 2012). The values for *cla, pala,* and *bla* were averaged per taxon and plot for every census date, leading to a longitudinal design with *elevation* and *taxon* as whole-plot factors (stratifying samples in independent groups), *time* as sub-plot factor (stratifying repeated measurements), and all possible interactions of the three factors. Due to the lack of normality of residuals and/or the lack of homoscedasticity, we used a rank-based, non-parametric analysis for longitudinal data as implemented in the R package nparLD v2.1 (Noguchi et al., 2012). To check for significant differences among elevations, we ran the same function ('f2.ld.f1') with a partial dataset, combining the two elevations to test against, again and adjusted *P*-values for multiple comparisons using Bonferroni correction. To check significant differences among taxa at each elevation we used the 'f1.ld.f1' function of the same R package.

In order to assess differences in rhizome dry weight *rdw,* we used a linear mixed effects model (R package nlme v3.1-108, Pinheiro et al., 2013) with *elevation* and *taxon* as fixed factors and *plot* as a random factor, while *rdw* was square root transformed to achieve normality of residuals. To ascertain heteroscedasticity for the values of the year 2012, we extended the linear mixed effects model with the 'varIdent' variance function to account for different variances among taxa. The maximal model contained the factors *taxon* and *elevation* plus the interaction of both. Model simplification was done as suggested by Crawley (2007), by merging factor levels that did not show significant differences. To test for correlation of *rdw* with the cumulative *pala* in mm^2, we used Spearman's rank correlation test.

3.4 Results

The damage of all plots at 600 m in 2012 through a larger animal was severe and hence data from that elevation and year were excluded from the analyses. Molluscs were identified as main herbivores. At all elevations, we found molluscs, their faeces, or mucus trails of molluscs on the transplants. Especially two weeks after transplantation, the number of molluscs was found being very high at 300 m (supplementary Figure A.2, appendix). In general, the number of molluscs was highest in most cases at the lowest elevation. Mollusc communities changed with elevation. While snails (determined as representatives of *Arianta abustorum, Trochulus striolatus danubialis,* and *Helix pomatia*) were found at lower elevations, slugs (determined as *Arion spec., Limax cineroniger*) could be found throughout the whole transect. Semislugs (e.g., *Semilimax kotulae*) were found predominantly at high elevations, where also insect herbivores occurred, yet to a negligible degree.

3.4.1 Leaf area parameters

In both experimental years, the leaf area parameters *cla, pala,* and *bla* were in general significantly influenced by elevation, time, and the interaction of both factors, but not by taxon assignment (Table 3.1). Herbivory, as measured by consumed leaf area *cla,* decreased with elevation (Figure 3.2a, b). Differences among taxa became obvious/significant at 900 m in 2011 and at 1200 m in 2012, with *S. hercynicus* preferred by herbivores over *S. ovatus* (2011/2012) and *S. germanicus* (2011), respectively. Hybrid genotypes, however, were neither preferred over their parental genotypes nor were they found being more resistant in both years. Either they showed intermediate values for loss of leaf tissue or they were found being damaged like the more intensively consumed parental genotype. Herbivore damage was highest in the first half of both observation periods (Figure 3.3a, b). Especially at the 300 m plots, almost all individuals were consumed entirely within 60 days after the start of the experiment in both years of the survey. The increase of damage caused by herbivores *(cla)* over time was found differing among elevations ($P < 0.03$), leading to significant elevation-time interactions (Figure 3.3a, b).

The photosynthetically active leaf area *(pala)* was found being lowest at 300 m in both years, highest at intermediate elevations (at 600 m in 2011 and at 900 m in 2012, respectively) and decreased again towards the highest elevations (Figure 3.2c, d). There were significant differences observable among taxa at 600 m upwards in 2011 and at 900 m in 2012, with *S. hercynicus* having significantly lower *pala* than the other taxa except for plots at 1200 m, where *S. ovatus* did not differ significantly from *S. hercynicus.* Hybrid taxa did not

Table 3.1: ANOVA-like table for the three estimated fitness parameters, consumed leaf area, photosynthetic active leaf area and brown leaf area, with two crossed between-plot factors *(elevation, taxon)* and one within-plot factor *(time)* for both experimental years. For between-plot factors the modified ANOVA-type statistic and for within-plot factors the ANOVA-type statistic was applied as implemented in the R package nparLD.

		2011				**2012**			
consumed leaf area *(cla)*									
	Source	**df1**	**df2**	**Statistic**	***P*-value**	**df1**	**df2**	**Statistic**	***P*-value**
between plots	Elevation	2.5	26.4	20.55	**<0.001*** **	1.5	13.7	72.06	**<0.001*** **
	Taxon	3.9	26.4	2.72	0.052	2.8	13.7	1.50	0.258
	Elevation:Taxon	9.1	26.4	1.16	0.357	4.3	13.7	1.3	0.433
within plots	Time	1.8		176.35	**<0.001*** **	1.6		251.77	**<0.001*** **
	Elevation:Time	2.2		9.6	**<0.001*** **	1.9		21.15	**<0.001*** **
	Taxon:Time	6.6		0.73	0.636	4.5		0.66	0.641
	Elevation:Taxon:Time	7.8		0.42	0.909	5.4		0.63	0.691
photosynthetic active leaf area *(pala)*									
	Source	**df1**	**df2**	**Statistic**	***P*-value**	**df1**	**df2**	**Statistic**	***P*-value**
between plots	Elevation	2.1	17.8	4.87	**<0.020***	1.9	16.9	24.54	**<0.001*** **
	Taxon	3.5	17.8	3.65	**<0.028***	2.7	16.9	1.90	0.157
	Elevation:Taxon	6.7	17.8	0.99	0.465	5.0	16.9	0.07	0.996
within plots	Time	2.7		442.58	**<0.001*** **	2.4		284.17	**<0.001*** **
	Elevation:Time	4.3		3.57	**<0.005****	3.3		8.20	**<0.001*** **
	Taxon:Time	9.1		2.10	**<0.025***	6.1		0.84	0.539
	Elevation:Taxon:Time	13.2		0.81	0.652	8.3		0.56	0.816
brown leaf area *(bla)*									
	Source	**df1**	**df2**	**Statistic**	***P*-value**	**df1**	**df2**	**Statistic**	***P*-value**
between plots	Elevation	2.6	25.0	56.98	**<0.001*** **	1.9	15.4	30.65	**<0.001*** **
	Taxon	3.4	25.0	1.64	0.201	2.8	15.4	0.30	0.814
	Elevation:Taxon	8.6	25.0	2.52	**<0.035***	49	15.4	0.42	0.827
within plots	Time	2.1		604.99	**<0.001*** **	2.7		369.42	**<0.001*** **
	Elevation:Time	4.6		28.86	**<0.001*** **	3.5		28.27	**<0.001*** **
	Taxon:Time	6.6		1.06	0.386	6.8		0.51	0.822
	Elevation:Taxon:Time	12.5		1.17	0.293	8.3		0.50	0.866

Levels of significance: $***P < 0.001$, $**P < 0.01$, $*P < 0.05$

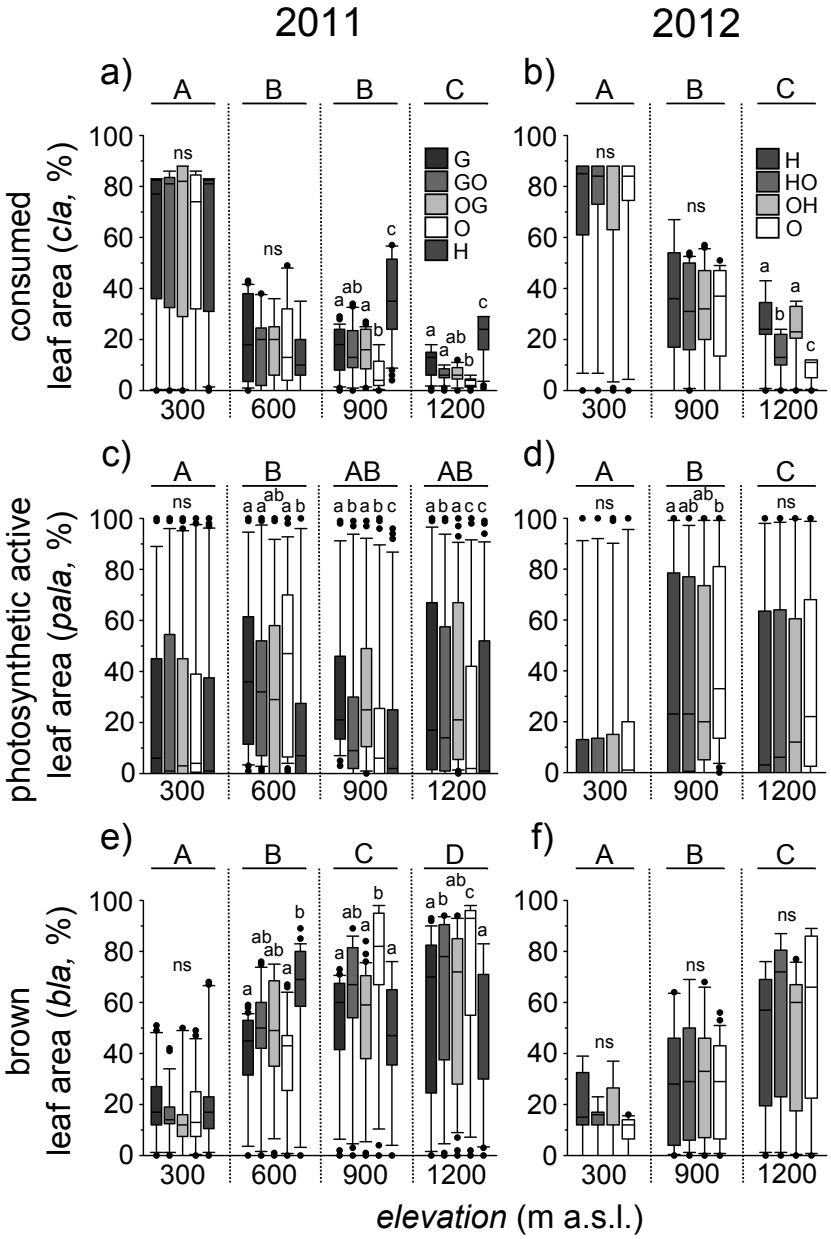

Figure 3.2: Impact of the between plot factors *taxon* and *elevation* on leaf area parameters for both observation periods. Capital letters indicate significant differences among elevations. Lowercase letters indicate significant differences among genotypes/taxa at the particular elevation. Abbreviations: G – *S. germanicus;* GO – *S. germanicus* × *S. ovatus;* OG – *S. ovatus* × *S. germanicus;* O – *S. ovatus;* H – *S. hercynicus;* HO – *S. hercynicus* × *S. ovatus;* OH – *S. ovatus* × *S. hercynicus.* In hybrid genotypes, the maternal parent is mentioned first.

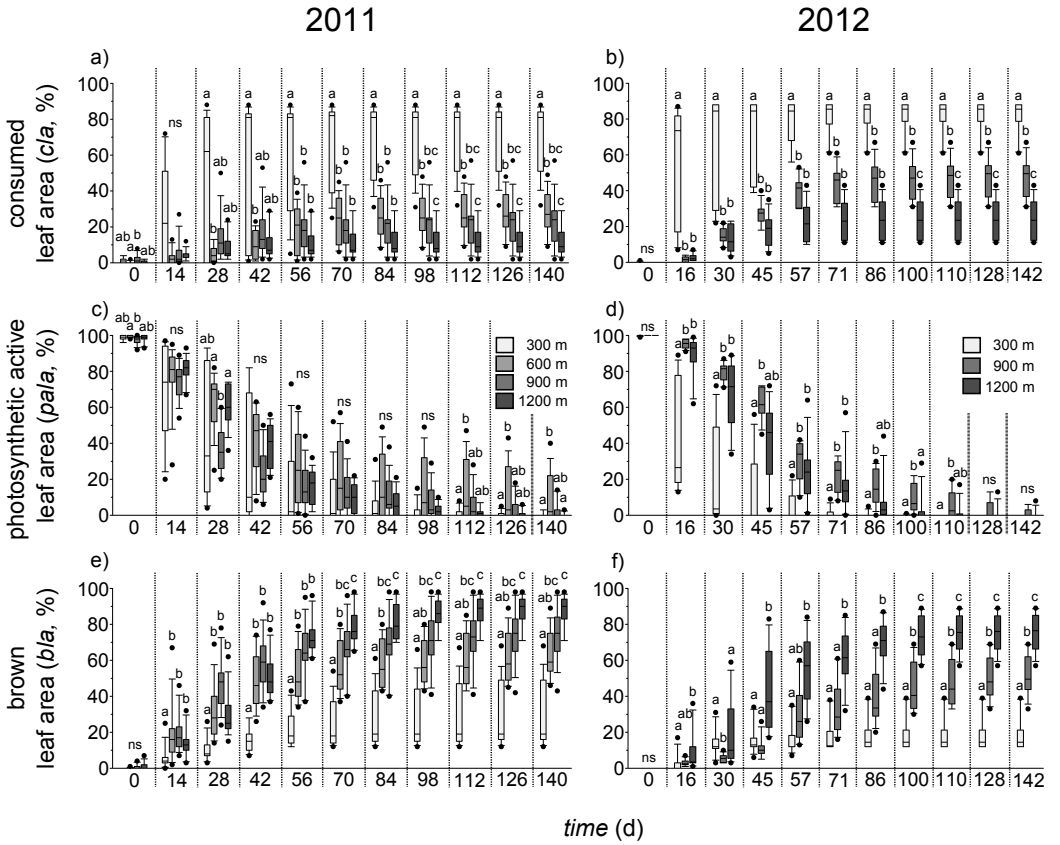

Figure 3.3: Impact of within plot factor *time* on leaf area parameters for both observation periods. Lowercase letters indicate significant differences among elevations at particular observation times.

show significantly lower or higher values for *pala* relative to their parental genotypes. In addition to significant elevation-time interactions between 900 and 1200 m in 2011 ($P < 0.001$) and among all elevations ($P < 0.003$) except 900 and 1200 m ($P = 0.072$) in 2012 (Figure 3.3c, d), we found a significant taxon-time interaction in 2011 (Table 3.1), when photosysnthetically active leaf area of *S. hercynicus* decreased faster than of *S. germanicus* ($P = 0.001$).

The withered, brown leaf area *(bla)* generally increased with elevation (Figure 3.2e, f) and showed a significant elevation-taxon interaction, with *S. ovatus* and *S. germanicus* exhibiting the lowest percentage and *S. hercynicus* the highest values for *bla* at the 600 m plots, while these relationships were found reversed at the 900 m and 1200 m plots (*S. hercynicus* = *S. germanicus* = *S. ovatus* × *S. germanicus* < *S. ovatus;* see Table 3.1). Hybrid genotypes were found being not significantly different from those parental genotypes with the lower *bla* or showed intermediate values. There were no statistically

Table 3.2: ANOVA table of the two minimal linear mixed effect models for the square root transformed rhizome dry weights *(rdw)* with two fixed factors *(elevation, taxon)* and one random factor *(plot)* for both experimental years. Factor levels that do not show a significant difference in intercept and slope were merged as indicated in brackets. For the 2011 model, the residual variance σ^2 is estimated as 0.047, and the variance for the random intercept d^2 is estimated as 0.007. For the 2012 model, the residual variance σ^2 is estimated as 0.005, and the variance for the random intercept d^2 is estimated as $\ll 0.001$. Abbreviations: G – *S. germanicus;* GO – *S. germanicus* × *S. ovatus;* OG – *S. ovatus* × *S. germanicus;* O – *S. ovatus;* H – *S. hercynicus;* HO – *S. hercynicus* × *S. ovatus;* OH – *S. ovatus* × *S. hercynicus.*

2011

	numerator df	**denominator df**	***F*-value**	***P*-value**
Intercept	1	261	435.72	**<0.001*** **
Taxon (G+GO ↔ OG+O+H)	1	261	9.31	**0.003****
Elevation (300+600 ↔ 900+1200)	1	10	13.84	**0.004****

2012

	numerator df	**denominator df**	***F*-value**	***P*-value**
Intercept	1	169	1772.54	**<0.001*** **
Taxon (H ↔ HO+OH+O)	1	169	12.49	**0.001****
Elevation (300 ↔ 900+1200)	1	7	42.89	**<0.001*** **
Taxon:Elevation	1	169	11.98	**0.001****

Levels of significance: $^{***}P < 0.001$, $^{**}P < 0.01$

significant differences in *bla* detectable among taxa at any elevation in 2012, but the mentioned trend for *S. ovatus* having larger proportions of leaf area withered than *S. hercynicus* at 1200 m was the same in 2012 as in the previous year. The withering process was strongest in the first half of both observation years and the increase of *bla* over time differed among all elevations ($P < 0.03$), except between 600 m and 900 m in 2011 ($P = 0.115$; Figure 3.3e, f).

3.4.2 Rhizome dry weight

In both years of the transplantation experiment, rhizome dry weight *(rdw)* was significantly influenced by taxon and elevation (Table 3.2). *rdw* was significantly higher for individuals growing in the two upper elevations in comparison to individuals growing at the lower elevations (Figure 3.4a, b). Hybrids exhibited different trends between the years: In 2011, hybrids approached their maternal

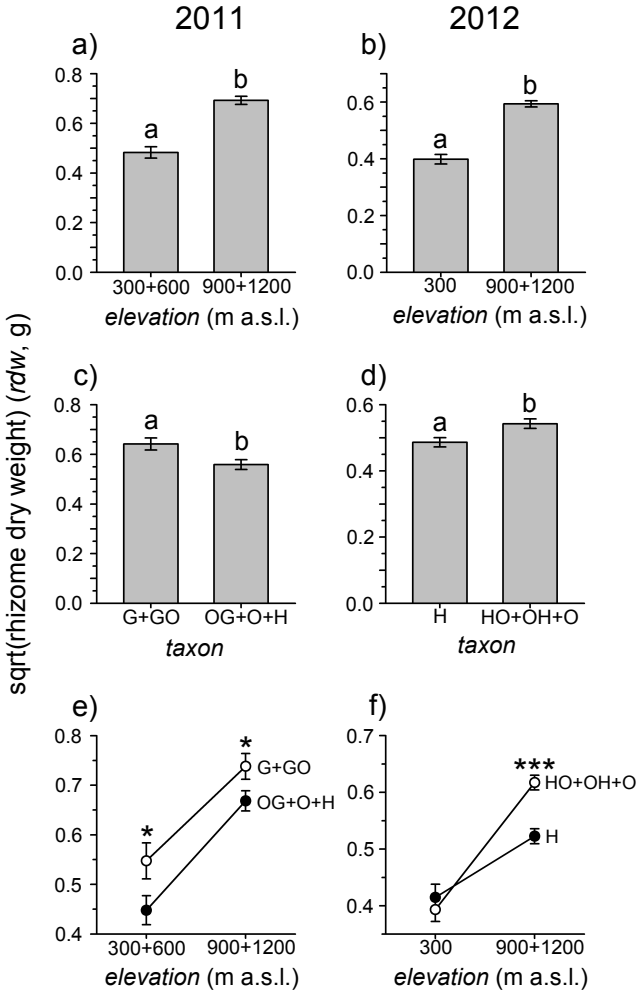

Figure 3.4: Impact of *taxon, elevation* and the interaction of both factors on the square-root transformed rhizome dry weight. Lowercase letters indicate significant differences. $^{***}P < 0.001$; $^{*}P < 0.05$. Abbreviations: G – *S. germanicus;* GO – *S. germanicus* × *S. ovatus;* OG – *S. ovatus* × *S. germanicus;* O – *S. ovatus;* H – *S. hercynicus;* HO – *S. hercynicus* × *S. ovatus;* OH – *S. ovatus* × *S. hercynicus.* In hybrid genotypes, the maternal parent is mentioned first.

parent with *S. germanicus* and GO-hybrids having a higher rhizome dry weight than *S. ovatus,* OG-hybrids, or *S. hercynicus* (Figure 3.4c), while in 2012, both reciprocal hybrid combinations (HO- and OH-hybrids) converged with *S. ovatus* showing higher *rdw* values than *S. hercynicus* (Figure 3.4d). Additionally in 2012, *S. ovatus* and hybrid taxa accumulated more *rdw* on the higher elevations than *S. hercynicus* as compared with the lowest elevation were both taxa groups showed no significant difference in *rdw,* together leading to a significant taxon elevation interaction. There was no such interaction in 2011 (Figure 3.4e, f). The correlation of *rdw* with cumulative *pala* was found to be significantly positive in both years (2011: $r_S = 0.44$, $S = 1.3e{+}6$, $P < 0.001$; 2012: $r_S = 0.70$, $S = 2.9e{+}5$, $P < 0.001$).

3.5 Discussion

Our results show that elevation and time play a primary role in influencing plant performance as assessed by leaf area-based parameters in the *Senecio nemorensis* syngameon. Taxon identity is secondary to that, but becomes more important with increasing elevation. Rhizome dry weight as a parameter determining successful propagation is mainly influenced by elevation and taxon identity and is significantly correlated with the size of the cumulative photosynthetically active leaf area of a plant.

3.5.1 Herbivory decreases with increasing elevation

As a general trend and irrespective of taxon identity, we found that the total leaf area consumed by herbivores decreased with elevation. This was already observed by Scheidel and Bruelheide (2001) for the species pair *S. hercynicus*-*S. ovatus* and was further described for other species of the Central European mountains with similar ecology and elevational range, like *Arnica montana* (Scheidel and Bruelheide, 1999a) and *Petasites albus* (Scheidel et al., 2003). Reasons for this trend could be either changes in the mollusc communities over elevation or the decrease of mollusc numbers and/or activity with increasing elevation. As far as the first point is concerned, Müller et al. (2009) could demonstrate that mollusc communities are varying considerably along the upper parts of our transect (i.e., in the parts between 640 m and 1440 m belonging to the Bavarian Forest National Park), with some representatives like the slug *Malacolimax tenellus* dominating at lower and the semislug *Semilimax kotulae* at higher elevations. Given the different body sizes, food preferences, and deterrent susceptibilities of mollusc species, the diversity of

mollusc communities could have a considerable influence on leaf damage along the elevational gradient of our present experimental design.

On the other hand, mollusc density and activity depends on the local climate, especially temperature, vapour pressure deficit (Crawford-Sidebotham, 1972), soil moisture (Nystrand and Granström, 1997; Martin and Sommer, 2004), and on the composition of the surrounding vegetation (Bonser and Reader, 1995; Scheidel and Bruelheide, 1999a; Loranger et al., 2014). Therefore, it is not surprising that plants at the lowest elevation site (300 m) near the Isar estuary with warm, wet climate and dense vegetation dominated by *Rubus caesius* showed very high mollusc damage of *Senecio* representatives. The lack of members of the *S. nemorensis* syngameon in these lowland riparian plant communities may also be a consequence of the high density and activity of mollusc herbivores here. Additionally, Cook et al. (2000) could demonstrate that slugs select food items according to deficient nutrients in previous diets, which may explain a probably over-proportional consumption of rare species (Cottam, 1985) like our transplants. At all other sites of our experimental gradient, natural *Senecio* populations were found in close proximity of the survey plots, demonstrating their climatic suitability and the acquaintance of herbivore communities with this plant group.

3.5.2 Rhizome dry weight is influenced by biotic and abiotic factors

Rhizome dry weight was found being positively correlated with the cumulated amount of the photosynthetically active leaf area *pala,* the latter in turn being influenced significantly by biotic (leaf area loss through herbivory) and abiotic (leaf area loss through withering) factors. Rhizomes at the lower elevations, where *pala* decreased rapidly due to herbivory, showed a lower dry weight than rhizomes from higher elevations, where plants remained green for a longer period an could accumulate more storage compounds in their rhizomes. Comparable situations were reported by Lubbers and Lechowicz (1989), who observed a negative influence of increasing defoliation on storage within plants, and by Scheidel and Bruelheide (2004), who observed that the rhizome dry weight of damaged *Petasites albus* plants was significantly lower compared to undamaged ones. Since the photosynthetically active leaf area *pala* was found decreasing fastest in *S. hercynicus* followed by *S. ovatus* and *S. germanicus* (see discussion below), the corresponding reversed trend of rhizome dry weight accumulated through the growth season being highest in *S. germanicus* and lowest in *S. hercynicus* is also influenced by taxonomy.

3.5.3 High palatability of *S. hercynicus* hampers advantage over the other two species in its native range

While at low elevation sites herbivore pressure was too high for observing taxon-selective feeding, from 900 m upwards we found significant differences in leaf consumption by molluscs. Here, *S. hercynicus* was the most consumed species followed by *S. germanicus* and *S. ovatus.* These results corroborate findings of Scheidel and Bruelheide (2001), who also observed a higher consumption of *S. hercynicus* compared to *S. ovatus* in a transplantation experiment. The role of pyrrolizidine alkaloids (PAs) as herbivore deterrents in the genus *Senecio* is well documented for generalists from different animal groups (vertebrates: Wiedenfeld and Edgar, 2011; insects: Macel, 2011; molluscs: Speiser et al., 1992; Dam et al., 1995; Aguiar and Wink, 2005), but indications of PA content in the three species under study vary considerably: While Herborg (1987) stated that the PA content of *S. ovatus* is 100 times smaller than of *S. hercynicus* and *S. germanicus,* Oberprieler et al. (2010, 2011) reported the lack of a significant difference between *S. hercynicus* (5.5 mg g^{-1} to 19.8 mg g^{-1}, mean 10.7 mg g^{-1}) and *S. ovatus* (3.8 mg g^{-1} to 19.3 mg g^{-1}; mean 9.4 mg g^{-1}) and an even reversed trend in a mixed stand of *S. ovatus* (5.6 mg g^{-1} to 52.9 mg g^{-1}; mean 15.7 mg g^{-1}) and *S. germanicus* (2.0 mg g^{-1} to 7.3 mg g^{-1}; mean 3.5 mg g^{-1}). Since the latter analyses were both carried out in hybrid swarms of the two species pairs and this may lead (through introgression) rather to an under- than to an overestimation of differences, we can certainly question Herborg's (1987) high PA content given for *S. germanicus* while higher PA contents in *S. hercynicus* compared to *S. ovatus* may be still conceivable. The stronger damage caused by molluscs in *S. hercynicus* despite similar or even higher PA contents may thus be explained by one or a combination of the following reasons: (a) Irrespective of PA content, *S. hercynicus* could be more palatable to molluscs than the other two species. As Mattson Jr (1980) and Mooney and Gulmon (1982) have pointed out, fast growing plant species often have high photosynthetic capacities and high nitrogen content. Being adapted to shorter vegetation periods at higher elevations, *S. hercynicus* demonstrably shows a faster development than the other two species (Herborg, 1987), which may account for a higher attraction to herbivores caused by its nutritiousness. However, while examples exist where fast growing species were preferred by herbivores (Mattson Jr, 1980; Price, 1991), there are others with contrary observations (Albrectsen et al., 2004). (b) Since Aguiar and Wink (2005) have shown that slugs are not deterred by alkaloids until exceeding a certain concentration, other chemical compounds than PAs or synergistic

effects of several compounds (Berenbaum and Neal, 1985) could be responsible for the preference of *S. hercynicus.* Anti-herbivore effects are also known for phenolics (especially phenolic glycosides and tannins; Maiorana, 1978; Fritz et al., 2001) and sesquiterpenes (Speiser et al., 1992), and these are also found in *Senecio* representatives (Fei et al., 2007; Meng et al., 2009). (c) Oberprieler et al. (2010, 2011) found that there are also qualitative differences among the three *Senecio* species in their PA composition. The higher damage by molluscs observed in *S. hercynicus* at high elevations may have been also caused by the adaptation of the local mollusc community to the species naturally growing at these elevational belts as compared to the two other 'alien' species with more exotic PA cocktails (Speiser et al., 1992).

While *S. ovatus* showed the lowest consumed leaf area at the highest elevations, this did not automatically come with a fitness advantage for this taxon. Comparing the brown leaf area of the three species, it becomes apparent that *S. ovatus* was more damaged by other factors than herbivory leading to a significant loss of photosynthetically active leaf area, at least in 2011. Climatic conditions are certainly very important determinants for the elevational niches of the three *Senecio* species under study; however, presently we lack detailed studies concerning the climatic requirements of the three taxa (temperature range, drought resistance, etc.). While the loss of photosynthetically active leaf area by withering in *S. ovatus* may point towards an elevated susceptibility to low temperatures at elevations typical for the more resistant *S. hercynicus,* this may also account for the observation of a reduced loss of leaf area through herbivores because the nutritional status of senescent leaves decreases (Mattson Jr, 1980; Hunter and Lechowicz, 1992; Lambdon et al., 2003) and so probably contributes to a lower preference by herbivores. Nevertheless, if the high susceptibility to low temperatures holds for *S. ovatus* it is questionable whether this taxon could reproduce successfully every year at high elevations. Introgressive hybridisation with *S. hercynicus* may therefore be an important mechanism for combining the advantage of the better herbivore resistance of *S. ovatus* with the possibly elevated frost resistance of *S. hercynicus.*

The third species, *S. germanicus,* which is naturally distributed at lower elevations and in warmer, continental climatic conditions, surprisingly shows low herbivore damage relative to *S. hercynicus* and low leaf area loss through withering relative to *S. ovatus,* thus resulting in a higher photosynthetic active leaf area (Figure 3.2c) and a higher cumulative rhizome weight (Figure 3.4c) compared to the two other species. Given that the PA content of *S. germanicus* plants is much lower compared to the other two species (Oberprieler et al. 2010, 2011), the observed relatively high resistance against herbivores could be best explained by the mechanical barrier to mollusc attacks provided by the hairy stems of this species compared to the glabrous ones of *S. hercynicus*

and *S. ovatus.* As Grime et al. (1970) have demonstrated trichomes on stems could effectively decrease mollusc movements on plants and accessibility of leaves, while presence or absence of trichomes on leaves being of lesser importance (Grime et al., 1968; Dirzo, 1980). While being favoured by reduced herbivore damage (relative to *S. hercynicus*) and seemingly low susceptibility to cold conditions (relative to *S. ovatus*), the retarded vegetative development of *S. germanicus* leading to its being the last flowering of the three taxa under common garden conditions (Herborg, 1987; own observations at the botanical garden of Regensburg University) may lead to flower or seed loss in the short vegetation periods of middle and high elevation conditions. This could be, therefore, the main obstacle for the invasion of this species into the eco-climatological domains of *S. ovatus* and especially *S. hercynicus.*

3.5.4 Artificial F_1 hybrids are neither advantaged nor disadvantaged in comparison to parental species

Following the classification proposed by Fritz (2001), the pattern of hybrid resistance to herbivores in our *Senecio* system rather corresponds to an additive or dominance pattern than to a susceptibility or resistance pattern. In both hybrid systems studied (i.e., *S. germanicus-S. ovatus* surveyed in 2011 and *S. hercynicus-S. ovatus* surveyed in 2012), we observed a trend of hybrids showing either leaf-damage values intermediate between the two parental taxa or showing values approaching the more intensively damaged parental species, this being *S. germanicus* in 2011 and *S. hercynicus* in 2012).

In the *S. germanicus-S. ovatus* hybrid system, the trend of hybrid genotypes (named *S.* ×*futakii* Hodálová) exhibiting intermediate values for the photosynthetically active leaf area *pala* is found resulting from two balancing tendencies: while hybrid individuals are significantly stronger damaged by herbivores than *S. ovatus* (Figure 3.2a), they tend to be less susceptible to withering than the latter taxon (Fig 3.2e). Ultimately, this seems to lead – irrespective of the differences caused by elevation influencing all taxa in a similar manner – to rhizome weights of the GO-hybrids being as high as in the maternal parent *S. germanicus,* whereas the OG-hybrids show reduced rhizome weights equivalent to their maternal parent *S. ovatus* (Figure 3.4c). In lower elevational ranges (200 m to 900 m; Herborg, 1987), where the two species naturally co-occur, herbivore pressure is quite high, but late frosts may develop frequently in troughs of the landscape. Here, the more effective phytochemical (presumably pyrrolizidine alkaloids) herbivore deterrent strategy of *S. ovatus,* together with its observed putative higher susceptibility to colder conditions and the intermediate status of hybrids in regards to both features

should eventually lead to a patchy distribution of the two parental species caused by the heterogeneity of climatic conditions (with *S. ovatus* winning over *S. germanicus* in warmer and thus mollusc-rich regions and the opposite situation in more continental and mollusc-poor parts of the landscape). Hybrid swarms formed by the two species, therefore, may constitute quite temporary systems in a labile balance that should develop into either pure *S. ovatus* or *S. germanicus* stands in dependence on microclimatological conditions. This is exactly what Oberprieler et al. (2011) found when morphologically, phytochemically, and genetically analysing populations of the *S. nemorensis* syngameon in the region of overlap between distribution ranges of *S. ovatus* and *S. germanicus* in SE Germany (Naab valley and Upper Palatinate Forest). In 14 out of 15 populations surveyed – even in locations where in 1988 hybrid swarms were found (Oberprieler, 1994) – either the one or the other species formed pure stands, and only a single (still enduring since 1988, but strongly asymmetrically introgressed) hybrid swarm was detected. However, while these observations of patchily distributed pure parental species and the lower than expected frequency of hybrid swarms could be plausibly explained by the surveyed vegetative fitness components, these preliminary discussions should be augmented by further information on other intrinsic and extrinsic factors jointly influencing overall fitness. The only data available so far on important intrinsic features are estimates of male fitness reported by Oberprieler (1989; cited in Oberprieler et al. (2011)) who found that individuals of *S.* ×*futakii* show only slightly lower (65-96%) pollen fertility values than the two parental species (87-99%).

While we lack information on further fitness components in the *S. germanicus-S. ovatus* hybrid system, Oberprieler et al. (submitted) could demonstrate for the *S. hercynicus-S. ovatus* system that hybrids (named *S.* ×*herborgii* C.Jeffrey) lack differences compared to the parental genotypes in generative (intrinsic) fitness components as pollen fertility, seed set, and germination rate. In the present study, we found that F_1 hybrids were significantly stronger damaged by herbivores than *S. ovatus* at 1200 m and either as strong as *S. hercynicus* (OH-hybrids) or intermediate between the two parental species (HO-hybrids). On the other hand, only non-significant trends were observed in respects to both the withered leaf area (H < OH < O < HO) and the resulting photosynthetically active leaf area (H < HO < OH < O), altogether leading, however, to a highly significant difference between low values of rhizome weight for *S. hercynicus* on the one hand and higher values for *S. ovatus* and the hybrid genotypes on the other gained until the end of the growth season (Figure 3.4d). Therefore, even in its natural habitat of clearings in high montane forests and subalpine meadows, *S. hercynicus* individuals seem to be outperformed by hybrid genotypes and *S.ovatus.* This easily explains the pattern of genetic

variation observed by Oberprieler et al. (2015) along an elevational transect between 750 m and 1300 m in the Bavarian Forest National Park, where all 11 populations surveyed along the transect were identified as being hybrid swarms with intense bilateral introgression. The simultaneous finding of a lack of genetic signatures that would indicate divergent selection along the transect, which would re-establish vertically stratified zones with pure parental genotypes (Oberprieler et al., 2015), is in accordance with the here described outperforming scenario caused by herbivores and argues for the appropriateness of the 'advancing wave model' (Fisher, 1937; Fritz, 2001) in this hybrid system. As a consequence, the rare *S. hercynicus* is under dire threat of being swamped by its spreading congener *S. ovatus* if global warming proceeds: (a) climatically induced range shifts of plant species will especially influence mountainous habitats (Grabherr et al., 1994; Lenoir et al., 2008; Thuiller, 2007; Truong et al., 2007) and will bring more and more populations of *S. hercynicus* in close contact with the ascending *S. ovatus,* (b) shifting plant phenologies in response to climate change (Cleland et al., 2007; Parmesan, 2006; Sherry et al., 2007; Walther et al., 2002) will promote gene flow between the two closely related, but phenologically isolated entities, (c) climatically induced changes in the herbivory regime, with quantitative and qualitative changes in mollusc communities (Müller et al., 2009), will favour hybrid genotypes and *S. ovatus* with their seen higher herbivore resistance over *S. hercynicus,* while finally (d) introgressively transferred frost resistance from *S. hercynicus* into *S. ovatus* via hybrids will further shift the genetic balance into the direction of the latter taxon.

3.5.5 Conclusions and outlook

Examples of elevational vicariance of closely related plant species may contribute interesting information for understanding the role of ecological factors during speciation processes (the emergence and growth of biodiversity) and the maintenance of organismal diversity (Abbott and Brennan, 2014). Additionally, comprehension of the loss of biodiversity caused by extinction through hybridisation requires detailed analyses of hybrid zones, including assessment of intrinsically and/or extrinsically mediated relative fitness estimates for parental and hybrid genotypes, of population demographical parameters, and of strength and extent of the selection regime in action along an ecological gradient (Ellstrand and Elam, 1993; Ellstrand et al., 1999; Levin et al., 1996; Wolf et al., 2001). In the present contribution, we have used a transplantation experiment along an elevational gradient to quantify the influence of herbivore (mollusc) communities and climatic parameters on the amount of photosynthetically active leaf area and gain of rhizome weight of three elevationally

vicariant members of the *Senecio nemorensis* syngameon (i.e., *S. hercynicus, S. germanicus,* and *S. ovatus)* and artificially crossed, reciprocal F_1 hybrids. We observed a general trend towards decreasing of herbivory with increasing elevation explainable by quantitative and qualitative changes in mollusc communities along the transect being affected by its eco-climatological and synecological correlates. Additionally, mollusc communities along the transect were found to damage the three *Senecio* taxa and their hybrids to different extents, which could be influenced by differences in herbivore composition, in phytochemical (pyrrolizidine alkaloids) and/or mechanical deterrent strategies of the plant taxa, or their differences in palatability. To disentangle effects caused by different mollusc taxa, pyrrolizidine alkaloid chemotypes, and other factors influencing palatability (e.g., tannin content), we are presently conducting food-choice ('cafeteria') experiments with all Senecio taxa and their hybrids on the one and different snail and slug species on the other side under controlled lab conditions.

Furthermore, we observed that photosynthetically active leaf area of *Senecio* plants was differentially reduced not only by herbivore activities, but also through withering; especially *S. ovatus* in the 2011 season suffered from leaf loss caused presumably by low temperatures at high elevations. Because we did not notice any similar detrimental effect in the 2012 season, we lack strong evidence for a consistent frost susceptibility of *S. ovatus* relative to the other two species. Due to the important influence of the amount of photosynthetically active leaf area on the overall viability of plants and their reproductive and propagational success (as assessed here in terms of rhizome weights), frost resistance of three *Senecio* species and their hybrids should be studied in more detail, especially in an experimental design excluding confounding effects by herbivores (by caging) and with a dense sampling of temperature fluctuations on plots (by using data loggers). As a matter of fact, effects like differential survival under winter conditions and gain or loss of rhizome weight and propagational power as important factors influencing overall fitness of genotypes of this clonally growing geophytes could be studied only in long-term transplantation experiments comprising several vegetation periods. These are also envisaged for the next phase of our project.

Finally, we have interpreted our findings arguing for the realisation of evolutionary trajectories towards a 'mosaic zone model' with patchily distributed pure and hybrid populations in the *S. germanicus-S. ovatus* system and towards a 'advancing wave model' in the *S. hercynicus-S. ovatus* system with the eventual swamping of *S. hercynicus* by *S. ovatus.* Besides the abovementioned long-term monitoring experiments, our argumentation could gain further plausibility by assessing additional intrinsic and extrinsic components of overall fitness of parental and hybrid genotypes, like male and female fertility,

gametophyte-sporophyte (in)compatibility reactions, seed set, germination rates, seedling survival, vegetative growth rates, etc. With the present studies on differential herbivory along an elevation transect and presently ongoing molecular genetic analyses of mixed stands, lab experiments on the influence of quantitative and qualitative phytochemical differences on herbivory, and a more detailed identification of pyrrolizidine alkaloid components, the members of the *S. nemorensis* syngameon will develop into a highly interesting model group for studying evolutionary consequences of plant hybridisation.

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CHAPTER 4

Phytochemical investigations and cafeteria experiments in three central European *Senecio* L. (Compositae, Senecioneae) species and their hybrids

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4.1 Abstract

Hybridisation is an important evolutionary process, leading to high qualitative and quantitative variability in secondary metabolites, which have a widely recognized function in defence against herbivores. The resulting differences in herbivore resistance between parental and hybrid taxa influence the trajectories of hybrid swarms and may lead to the maintenance or breakdown of species boundaries. In the present study, we investigate phytochemical patterns in three Central European representatives of the *Senecio nemorensis* syngameon (i.e., *S. germanicus, S. hercynicus,* and *S. ovatus*) that show mutual hybridisation in their range overlaps. We further investigate selective consumption among the pure species, the pure species and their hybrids, and the effect of pyrrolizidine alkaloids (PA) and tannins on the food acceptability in food choice ('cafeteria') experiments. Our results show significant qualitatitve and quantitative differences among the three species. Artificial F_1 hybrids of the two investigated plant systems *S. germanicus-S. ovatus* and *S. hercynicus-S. ovatus* mainly expressed an additive PA pattern, but also a novel compound could be found in the reciprocal hybrids of the first system. PA content was highest in *S. hercynicus,* intermediate in *S. ovatus* and lowest in *S. germanicus.* In contrast, tannin content was lower in *S. hercynicus* compared to *S. ovatus* and *S. germanicus.* Cafeteria experiments revealed significant species-specific preference by two mollusc species (*Arion fuscus* and *Trochulus striolatus danubialis*). However, consistent negative correlations of the total PA content and consumed leaf area only existed for the snail, but not for the slug species. As soon as reciprocal hybrid taxa were added to the cafeteria designs, the species-specific consumption disappeared, which could lead to an advantage for the previously preferred species. We assume that for PAs, there is no general food deterrent effect, and other taxon-specific features might be responsible for the selective consumption. Additionally, there was no significant effect of tannin content in neither experimental design.

4.2 Introduction

Until the late 20th century, secondary metabolites (SMs) were considered to be waste and detoxification products of the primary metabolism (Mothes, 1955; Luckner, 1990). Nowadays, however, it is generally accepted that they represent an important interface for the interaction of plants with their environment, with defence being just one function among others (Hartmann, 1996). For several SMs, a function as antifeedant could be demonstrated (e.g., alkaloids: Aguiar and Wink, 2005; Cheng et al., 2011b; cyanogenic glycosides: Cooper-Driver and Swain, 1976; Dirzo and Harper, 1982; sesquiterpenes: Ahern and Whitney, 2014; Hägele et al., 1996; tannins and phenolic glycosides: Forkner et al., 2004; Fritz et al., 2001).

In respects to hybridisation, secondary chemistry was found to be qualitatively and quantitatively variable in hybrids and its impact on herbivory and consequently its influence on the trajectories of hybrid swarms is an interesting field of research (see reviews by Cheng et al., 2011a and Orians, 2000). As Cheng et al. (2011a) pointed out in their meta-analysis of 1,112 secondary metabolites (SM), in most cases (70.3%) hybrids expressed the SMs of their parents. But chemical compounds also may disappear in hybrids (24.2%) or completely new SMs are formed (5.5%), although this is less common in first generation hybrids (Orians, 2000, and references therein). In a second meta-analysis, Cheng et al. (2011a) investigated the quantitative variation of SMs in F_1 hybrids relative to their parents. The concentration in F_1 hybrids was found being similar to one or both parents or intermediate in most cases (79.8%). The proportion of over- or underexpressed SMs in hybrids compared to their parents was low (11.5% and 8.7%, respectively). However, it is not possible to infer the pattern of hybrid resistance to herbivory from the patterns of quantitative SM variation. For example, Cheng et al. (2011a) figured out that hybrids are more often susceptible to herbivores than could be expected from the frequency of underexpressed SMs. Nevertheless, five main patterns can be defined in terms of herbivore resistance in hybrids. (1) no difference pattern: resistance of hybrids and parental species is equal to each other; (2) additive pattern: hybrids show an intermediate resistance relative to the differing parental genotypes; (3) dominance pattern: hybrid resistance is similar to one of the parental genotypes, but significantly different from the other genotype; (4) susceptibility pattern: hybrids are more susceptible to herbivores than parental genotypes; (5) resistance pattern: hybrids show higher herbivore resistance than parental genotypes (Fritz, 1999). On the basis of these patterns, different predictions can be made about the evolution of the genetic constitution of a hybrid swarms. If hybrids correspond to the susceptibility pattern, they could limit the abundance of herbivores on the parental genotypes

(hybrids-as-sinks hypothesis; Whitham, 1989). As a consequence, the genetic swamping of these genotypes is less probable. In contrast, if hybrids are more resistant or are as resistant as the parental genotypes, and additionally have higher or equal overall fitness values than the parental genotypes, introgression becomes possible and the final swamping of the parental genotypes is probable.

Pyrrolizidine alkaloids (PA) and tannins are two classes of very diverse secondary metabolites. There are over 660 PAs and PA-*N*-oxide derivatives known from more than 6,000 angiosperm species (Fu et al., 2004), from which 95% were identified from four plant families: Compositae, Boraginaceae, Leguminosae, and Orchidaceae (Hartmann and Witte, 1995). Within the Compositae, PAs have their main occurrence in the tribe Senecioneae, with c. 190 different compounds identified from c. 300 Senecioneae species (Langel et al., 2011, and references therein). Tannins are widely distributed within dicots (Bate-Smith, 1962). Bate-Smith (1962) found in his study of over 1,000 species within 180 different families that the distribution of tannins is not equal among the investigated families, and that tannins tend to be more common in woody than in herbaceous plants. Concerning the function of secondary metabolites, which is – amongst others – the defence of plants against other organisms (Hartmann, 1996), for each of the above mentioned two classes, deterrent and/or detrimental effects on vertebrate and invertebrate herbivores were demonstrated (PAs: Candrian et al., 1984; Dreyer et al., 1985; Grecco et al., 2011; Harper et al., 1985; Hägele and Rowell-Rahier, 2000; Mattocks, 1986; Wei et al., 2015; tannins: Barbehenn and Constabel, 2011; Bernays et al., 1980; Fritz et al., 2001; Swain, 1977). But there is also a number of specialists that adapted to these compounds (Barbehenn and Martin, 1992; Bernays et al., 2004; Boppré, 1986; Edgar et al., 1976; Schultz, 1989).

The three central European representatives of the *Senecio nemorensis* syngameon are vertically vicariant species. *Senecio germanicus* Wallr. subsp. *germanicus* prefers lower elevations and a more continental climate, *S. ovatus* (P.Gaertn., Mey. & Scherb.) Willd. subsp. *ovatus* colonises intermediate ones with more oceanic conditions and *S. hercynicus* Herborg var. *hercynicus* grows in high elevations with a high montane or subalpine climate. Due to incomplete crossing barriers, particularly due to partial overlap in flowering time, they are able to hybridise with each other in geographically overlapping zones. Based on morphological, phytochemical (i.e., PAs), and genetic data, hybridisation was detected in the *S. germanicus*-*S. ovatus* species pair and the *S. hercynicus*-*S. ovatus* species pair, with hybridisation occurring to different extents (Oberprieler et al., 2010, 2011; Raudnitschka et al., 2007). Concerning herbivory in a *S. hercynicus*-*S. ovatus* hybrid swarm, Oberprieler et al. (submitted) found a significant influence of the genetic constitution, phenology, and PA content on the consumption of leaf material by herbivores. *Senecio*

hercynicus-like as well as early-flowering individuals were more damaged than hybrid genotypes and *S. ovatus*-like or late-flowering individuals. Surprisingly, there was a trend of *S. hercynicus*-like individuals, showing higher PA content, to be more damaged. This is in line with the observation of transplantation experiments, were Bog et al. (submitted; chapter 3) found that *S. hercynicus* sustains stronger damage by herbivores in higher elevations than *S. ovatus* or *S. germanicus.* Additionally, *S. germanicus* suffers stronger damage than *S. ovatus.* Hybrids of either species pair showed an intermediate behaviour in comparison to their parental species (Bog et al., submitted; chapter 3).

In the field, many abiotic and biotic factors can influence the outcome of experiments. Therefore, the present study investigates the selective consumption of the three *Senecio* species and their hybrids under controlled conditions in food choice experiments with two generalist herbivores (molluscs) that could be found in plots of the aforementioned transplantation study. The following two questions are at the focal point of the present study. (1) Are there significant qualitative and quantitative differences in the deterring secondary metabolite content (i.e., PAs and tannins) among the three parental species and in comparison to their artificial F_1 hybrids? (2) Are there significant differences in consumption among the parental species and compared to their F_1 hybrids in food choice experiments and, if so, can this be explained either by PA or tannin content?

4.3 Material and methods

4.3.1 Study species

Senecio germanicus, S. hercynicus, and *S. ovatus* are tetraploid ($2n = 40$), insect-pollinated, perennial geophytes and reported to be self-incompatible (Herborg, 1987; pers. obs. 2011). They are capable of producing large numbers of wind-dispersed fruits (achenes) and also propagate vegetatively by formation of stolones. All three are members of plant communities typical of succession stages after natural or anthropogenic disturbances (e.g., forest clearings). Conspicuous differences in morphology between the study taxa concern the leaf bases of the upper cauline leaves, which are sessile in *S. germanicus* and *S. hercynicus* and petiolate in *S. ovatus.* Further, the dimension and shape of the capitula is discriminating the three taxa. *Senecio germanicus* and *S. hercynicus* are characterised by larger capitula with a higher number of tubular/disc florets and involucral bracts than *S. ovatus.* In addition, in *S. germanicus* subsp. *germanicus* the supplementary involucral bracts are furnished with long eglandular hairs and also the lower part of the stem

possesses long eglandular hairs. *Senecio hercynicus* is characterised by the possession of glandular hairs on leaves, the upper part of the stem and on the supplementary bracts of the capitulum. In contrast to these two species, in *S. ovatus* only articulate hairs are found.

When growing in a common-garden experiment at the University of Regensburg, Bavaria (Germany), the three species exhibit considerable differences in phenology (pers. obs. 2011, 2013): although the shoots emerge nearly simultaneously around the middle of April, *S. hercynicus* grows much faster than the other two species and flowers around four weeks earlier than *S. ovatus,* while *S. ovatus* in turn flowers around two weeks earlier than *S. germanicus.* The average flowering time of *S. hercynicus* is in general between June and July, for *S. ovatus* between July and August and for *S. germanicus* between July and September. However, isolation by flowering time is not complete and hybridisation is possible, with F_1 hybrids of *S. germanicus-S. ovatus* and *S. hercynicus-S. ovatus* crosses showing intermediate flowering times (pers obs. 2013).

4.3.2 Plant cultivation and crossings

Parental individuals were collected before commencement of flowering and transplanted into pots in late spring and early summer 2010. We used plants from populations, for which the morphological and/or genetic constitution had been analysed before and hybridisation with one of the other species could be excluded. *Senecio germanicus* was collected near Penk-Distelhausen, Bavaria, Germany (population 9 of Oberprieler et al., 2011; henceforth referred to as 'G1'), *S. hercynicus* from Eschacher Wald near Kempten, Bavaria, Germany (population 1 of Oberprieler, 1989 and Oberprieler, 1994; henceforth referred to as 'H') and *S. ovatus* near Unterlichtenwald, Bavaria, Germany (henceforth referred to as 'O1'). Seeds of pure species were collected in the same populations later in that year. Because seed production in O1 and G1 was very low, we additionally collected seeds of *S. ovatus* and *S. germanicus* in 2012 from further populations near Falkenstein ('O2') and Leinschlag ('G2'), Bavaria, Germany.

Crosses among the three species were made in the botanical garden of the University of Regensburg. To prevent insect-pollination, inflorescences were covered with self-made bags from commercially availabe pollen gauze. *Senecio ovatus* and *S. germanicus* were hand-pollinated between middle of July and beginning of August 2010. *Senecio ovatus* and *S. hercynicus* were hand-pollinated between end of June and beginning of July 2011. To increase yield of hybrid seed, previously harvested and frozen (-18°C) pollen of *S. hercynicus* was used to hand-pollinate *S. ovatus* for an extended period until middle of July 2011. Seeds were harvested in the beginning of September 2010 (*S. germanicus-*

S. ovatus crosses) and in the middle of August 2011 (*S. hercynicus-S. ovatus* crosses) and stored at 5°C under dark and dry conditions. To adjust all individuals to a homogenous starting point in their development, the pericarp and testa of achenes were removed and the embryos were kept on moistened filter paper in Petri dishes under greenhouse conditions (20°C/12°C, 14 h day / 10 h night) for about one week. Seed preparation for the first experimental year (*S. hercynicus, S. ovatus, S. germanicus,* and the reciprocal crosses of the two latter species) started by the end of November 2010 and for the second experimental year (*S. ovatus, S. hercynicus,* and their reciprocal crosses) in the middle of January 2012. Seeds for preparation were chosen randomly. One week old plants were transplanted into seed trays in a 4:2:0.5 compost : Einheitserde® Classic (Sinntal – Altengronau, Germany) : sand mixture. After about seven weeks, they were planted into 12-cm pots. Green house conditions and soil mixture were kept constant during growing. To control plant pests, we used glue panels against sciarid flies and plant protection oil against aphids. Plants were transferred to experimental fields of the botanical garden for acclimatisation in the middle of March in both years. To check whether the crosses were successful (i.e., hybrid genotypes were formed), we used the genotyping method as described in Bog et al. (submitted, chapter 3)

4.3.3 Phytochemical investigations

After punching out leaf discs for the cafeteria experiments (see below), the remaining leaf area was silica-gel-dried and the PAs were extracted according to the protocol of Oberprieler et al. (2010), which is a modified version (Hagen, 2003) of the acid-base extraction method described by Hartmann and Toppel (1987). We added 50 µg of Heliotrin (Latoxan, Valence, France) as an internal standard and analysed the samples using gas chromatography through a 30-m-long factorFOUR VF-1ms column (Varian, Darmstadt, Germany). Conditions (injector temperature of 240°C; temperature program: initial step 120°C for 5 min, followed by 120 to 220°C with an increment of 5 °C min^{-1} and a final step of 20 min at 220°C; split-less injection, followed by a 1:50 split after 1.5 min; carrier gas He 2.0 ml min^{-1}, makeup N_2 28 ml min^{-1}; detector temperature of 280°C) broadly followed the protocol given by Witte et al. (1993) but were improved for the present extracts after comprehensive testing. Runs were made on a CP-3900 gas chromatograph (Varian) and controlled by the programme Galaxie Chromatography Workstation v1.8.508.1 (Varian). The same software programme was also used to quantify peak areas. Selected extracts were also analysed by GC-EIMS (70 eV) to identify peaks representing PAs by the operating unit 'Zentrale Analytik' (University of Regensburg, Regensburg, Germany). Only compounds matching the typical fragmentation pattern of

PAs were included in the investigation. Compound identification was done by Dr. T. Beuerle (TU Braunschweig, Braunschweig, Germany). Not all leaf discs from the cafeteria experiment were analysed for their PA content, as we selected only ten Petri dishes from each experimental design (see below) for analysis.

Tannins were analysed according to the hide powder method as described in European Pharmacopoeia (2011). The extraction was improved for our samples but the measurement of total polyphenol content and the content of polyphenols not adsorbed by hide powder, the difference being the content of tannins, was done as described (European Pharmacopoeia, 2011). For the investigated plants, 250 mg of silica-dried leaf material were milled to fine powder using a vibratory disc mill RS1 (Retsch, Haan, Germany). We added 50 ml of boiling MeOH/H_2O (50:50 v/v) and kept the suspension at 80-90°C for 30 min. Then, the suspension was cooled to room temperature under running water, diluted to 100 ml with deionized water, and centrifuged at 3,000 rpm for 5 min. We additionally filtered the liquid through filter paper. To calibrate our measurements, we used 50 mg chlorogenic acid (Roth, Karlsruhe, Germany) as standard. The percental content of tannins expressed as chlorogenic acid equivalents was calculated from the expression:

$$\frac{25(A1 - A2)m^2}{(A3 * m1)}$$

where, $A1$ = absorbance of total polyphenols,
$A2$ = absorbance of polyphenols not absorbed by hide powder,
$A3$ = absorbance of chlorogenic acid,
$m1$ = mass of the sample to be examined (g),
$m2$ = mass of chlorogenic acid (g).

4.3.4 Cafeteria experiments

Cafeteria experiments were carried out with the slug *Arion fuscus* (O.F.Müller 1774) and the snail *Trochulus striolatus danubialis* (Clessin 1874). Different *Senecio* taxa were tested against each other in four experimental designs for each mollusc, respectively: a) *S. germanicus* against *S. ovatus,* b) *S. hercynicus* against *S. ovatus,* c) *S. germanicus* against *S. ovatus* and their reciprocal F_1 hybrids and d) *S. hercynicus* against *S. ovatus* and their reciprocal F_1 hybrids. For the experimental designs a) and b) plant material was taken from individuals, which were transplanted from the field, while for the experimental designs c) and d) predominantly plant material was taken from individuals grown in the same year when the experiments were performed. Experiments were carried out in 9 cm Petri dishes laid out with three layers of paper tissue

soaked with 5 ml of water. Leaf discs (16 mm in diameter) were mounted with a needle. For each design, 30 pairs or quartets of leaf discs were investigated with three repetitions, except experimental designs a) and b) for *A. fuscus* (five repetitions), and arithmetic mean values were calculated. Experiments were run during June 2011 and June until mid August 2012 over night (16 h) for the slug *Arion fuscus* or 92 h for the snail *Trochulus striolatus danubialis* in a shady place of the botanical garden. After the experiments, leaf discs were scanned and the consumed area was determined using the programme ImageJ v1.45s (Wayne Rasband, downloadable at http://rsbweb.nih.gov/ij/).

4.3.5 Statistical data analysis

Statistical analyses were carried out with R v2.14.2 (R Development Core Team, 2012). In order to assess differences in total PA concentration *(PA_{tot})* and tannin content among species and taxa, we used a generalised least squares model (R package nlme v3.1-108, Pinheiro et al., 2013). PA content was log transformed after a small value of 0.001 was added and tannin content was square or cube root transformed to reach normality of residuals. We used the varIdent variance function to account for different variances among taxa. Multiple comparisons were done using the Tukey test as implemented in the R package multcomp (Hothorn et al., 2008).

After calibration of peak areas of gas chromatograms according to the internal standard of heliotrine and the initial net weight of leaf material, we used the absolute quantity of each of the nine PAs selected for the present analyses in each OTU to perform a detrended correspondence analysis (DCA) using the R package vegan v2.0-7 (Oksanen et al., 2013). DCA was employed to account for the 'horseshoe effect' observed when analysing the data in a principle component analysis.

In order to evaluate whether taxon identity or phytochemistry has a significant effect on the consumption by the molluscs we used a linear mixed effects model (R package nlme v3.1-108, Pinheiro et al., 2013). To account for the dependent sampling design, we included the factor *petri dish* as a random factor. Again, PA content was log transformed after a small value of 0.001 was added and the *consumed leaf disc area* was square or cube root transformed to reach normality of residuals. We used the 'varIdent variance' function to account for different variances among taxa, again. Three independent models were run with *taxon,* PA_{tot} and tannin concentration *(tannins)* as independent factors, because *taxon* and PA_{tot} as well as *tannins* were highly correlated and the sampling for PA_{tot} and *tannins* was too different, leading to a high number of missing values, which cannot be handled by the model. A tentative model

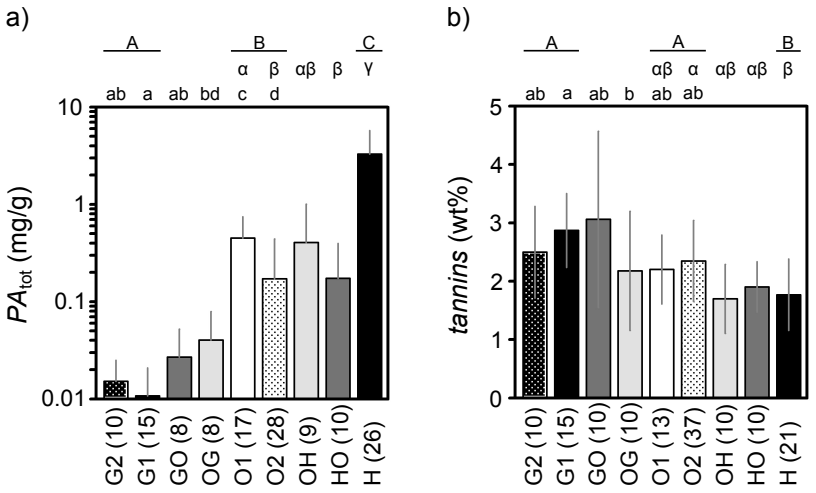

Figure 4.1: Differences in a) PA_{tot} and b) tannins among the investigated taxa. Capital Latin letters – significant differences among pure species; lower-case Latin letters – significant differences among taxa of the GO-system; lower-case Greek letters – significant differences among taxa of the HO-system.

run with a reduced data set did not give deviating results or a significant interaction term between PA_{tot} and *tannins.*

4.4 Results

4.4.1 Differences in phytochemistry among taxa

We found significant differences among pure species in the content of the two investigated compound classes (Figure 4.1). The total PA content of *S. hercynicus* (mean: 3.285 mg g^{-1}, 0.190 mg g^{-1} to 7.765 mg g^{-1}, SD: 2.442 mg g^{-1}, $N = 26$) was significantly higher than in *S. ovatus* (mean: 0.278 mg g^{-1}, 0.004 mg g^{-1} to 1.099 mg g^{-1}, SD: 0.307 mg g^{-1}, $N = 45$), and that in turn was significantly higher than in *S. germanicus* (mean: 0.013 mg g^{-1}, 0.001 mg g^{-1} to 0.042 mg g^{-1}, SD: 0.010 mg g^{-1}, $N = 25$). While hybrids of the *S. germanicus-S. ovatus* system showed concentrations similar to the parental *S. germanicus* population (GO) or intermediate concentrations to either parental population (OG), hybrids of the *S. hercynicus-S. ovatus* system showed concentrations similar to *S. ovatus* (OH) or even significant lower concentrations than either parental population (HO). The tannin concentration did not differ between *S. germanicus* (mean: 2.72 wt%, 1.67 to 4.60 wt%, SD: 0.71 wt%, $N = 25$) and *S. ovatus* (mean: 2.31 wt%, 0.91 - 4.20 wt%, SD: 0.67 wt%, $N = 50$), but it was significant lower for *S. hercynicus* (mean: 1.77 wt%, 0.65 to 2.75 wt%,

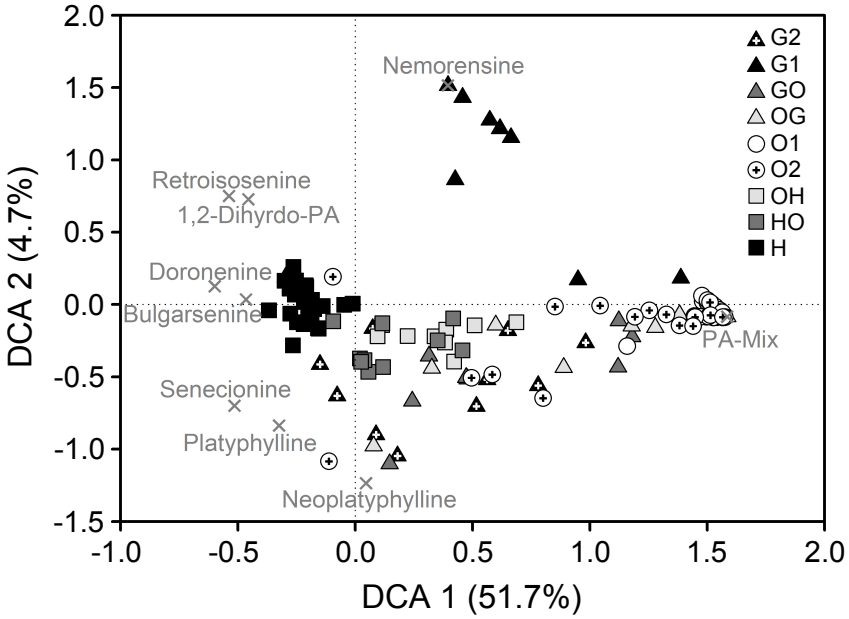

Figure 4.2: Detrended correspondence analysis (DCA) based on the absolute concentration of each of the nine selected PAs in the gas chromatographic analysis.Thr proportions of explained variation are given on the axes.

SD: 0.61 wt%, $N = 21$) compared to the other two species. Hybrids of the *S. germanicus-S. ovatus* system had the same tannin concentration as the maternal populations by trend, and were not significantly different from each other. In the *S. hercynicus-S. ovatus* system, hybrids did not show significant differences to their parental populations, while parental populations were also found not being different.

The result of the detrended correspondence analysis is shown in Figure 4.2. According to the qualitative and quantitative PA composition, the three parental populations are arranged in a triangle with a few exceptions for *S. germanicus*. Hybrids of the *S. hercynicus-S. ovatus* system show an intermediate position relative to their parental populations with a slight shift towards *S. hercynicus*. Contrary to our expectations, hybrids of the *S. germanicus-S. ovatus* system are found near the *S. hercynicus-S. ovatus* hybrids, what suggests the assumption that there is some phytochemical novelty within this system. Further, the results show that there are also differences in PA composition among different populations of the same species.

These findings become more clear in Figure 4.3, which shows the relative PA concentrations of the investigated taxa for each of the nine selected PA peaks. In particular, the 1,2-dihydro-PA peak can be found in *S. hercynicus* and all hybrid taxa from either system, but it is neither existent in the two *S. ovatus*

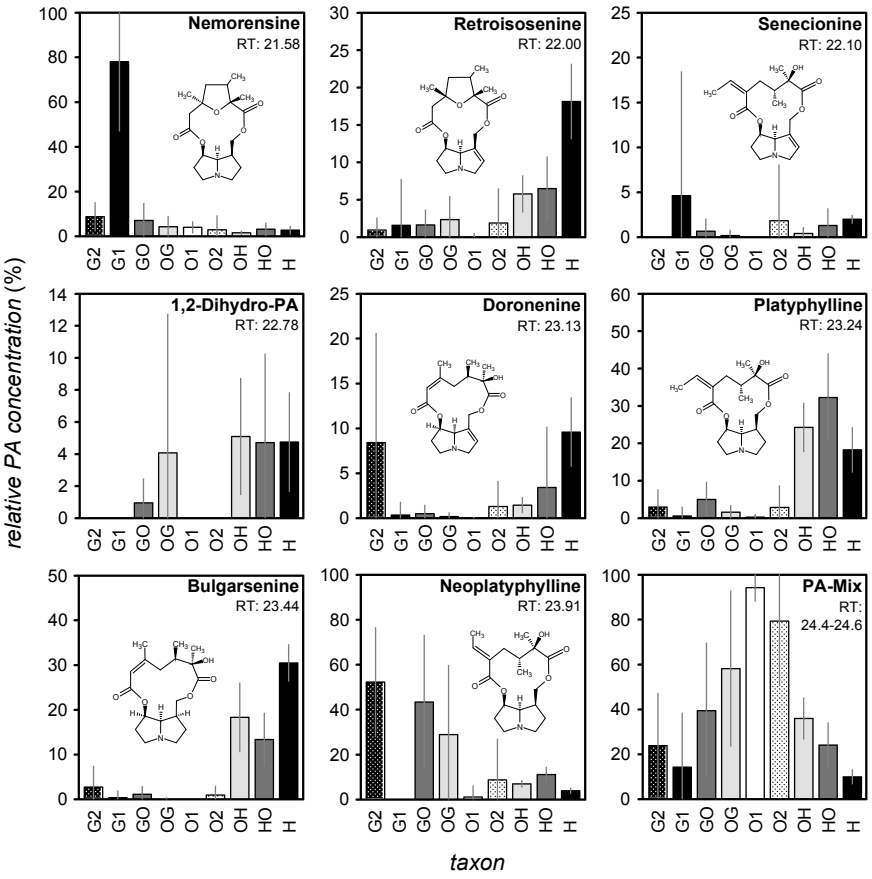

Figure 4.3: Relative PA concentration of the nine selected PAs for the investigated taxa. For each PA peak the retention time (RT in min) is given and the structure for identified PA peaks is shown. PA-Mix represents two PAs (triangularine isomer and neoplatyphylline isomer), which could not be separated by our analysis. As a consequence, for these a RT range is given. The RT for the heliotrine standard was at 18.76 min.

populations nor the two *S. germanicus* populations. Further, *S. hercynicus* and its hybrids with *S. ovatus* show all PA peaks, while the latter species is mainly characterised by the PA-Mix peak, that comprises a triangularine isomer and a neoplatyphylline isomer, but which could not be separated from each other in our analysis. The most distinct differences in population-wise PA composition can be found in *S. germanicus*. The overall low PA content of G1 is mainly composed of nemorensine, while the main PAs of G2 are neoplatyphylline and the two PAs from the PA-Mix peak.

4.4.2 Relationship between phytochemistry and herbivory

In the cafeteria experiments, the snail *Trochulus* showed an overall lower leaf disc consumption than the slug *Arion*. However, either mollusc species differed in the consumption of the offered taxa. But this holds only true for designs, in which the two pure parental taxa were offered to the molluscs. As exemplified in the Figures 4.4b, 4.4d, 4.5b and 4.5d, the consumption bias disappears when four taxa were offered simultaneously. Figures 4.4a and 4.4c show that *S. germanicus* is preferred over *S. ovatus* by either mollusc species in the *S. germanicus*-*S. ovatus* system. In the *S. hercynicus*-*S. ovatus* system, *S. ovatus* is preferred over *S. hercynicus* by *Trochulus* (Figure 4.5c) while, contrary to our expectations, *Arion* preferred *S. hercynicus* over *S. ovatus* (Figure 4.5a), having a higher PA_{tot} than *S. ovatus*. This leads to a surprisingly significant positive correlation of PA_{tot} and leaf disc consumption (Figure 4.5e). Interestingly, this positive correlation turns into a negative correlation, when four leaf discs were offered. In this experiment, *S. hercynicus* individuals were less consumed than *S. ovatus* individuals by trend (Figure 4.5f). However, in most cases, there was a significant negative correlation of PA_{tot} and leaf disc consumption (Figure 4.4e, 4.4f, 4.4h, 4.5g, 4.5h) and in two cases (Figure 4.4g, 4.5f) no significant correlation was found. Concerning the tannin content, no relationship could be detected with the leaf disc consumption for either experimental design (Figure 4.4i-k and Figure 4.5i-k).

4.5 Discussion

4.5.1 Differences in phytochemistry among the parental species

Our results show that the parental species exhibit quantitative and qualitative differences in their phytochemistry, especially in their PA composition. Already Herborg (1987) investigated the patterns of PAs quantitatively and qualitatively of the three species used in the present study. He found the content of *S. ovatus* to be 100 times lower than in *S. germanicus* and *S. hercynicus*. We cannot confirm this finding with our results, where the PA content of *S. hercynicus* was about 10 times higher than in *S. ovatus*, and that in turn was about 20 times higher than in *S. germanicus*. On the basis of qualitative PA patterns, Herborg (1987) grouped *S. germanicus* and *S. hercynicus* to one and *S. ovatus* to another chemotype. We here would suggest a separate chemotype for *S. germanicus*, too. The nine selected PA peaks were present in all individuals of *S. hercynicus*,

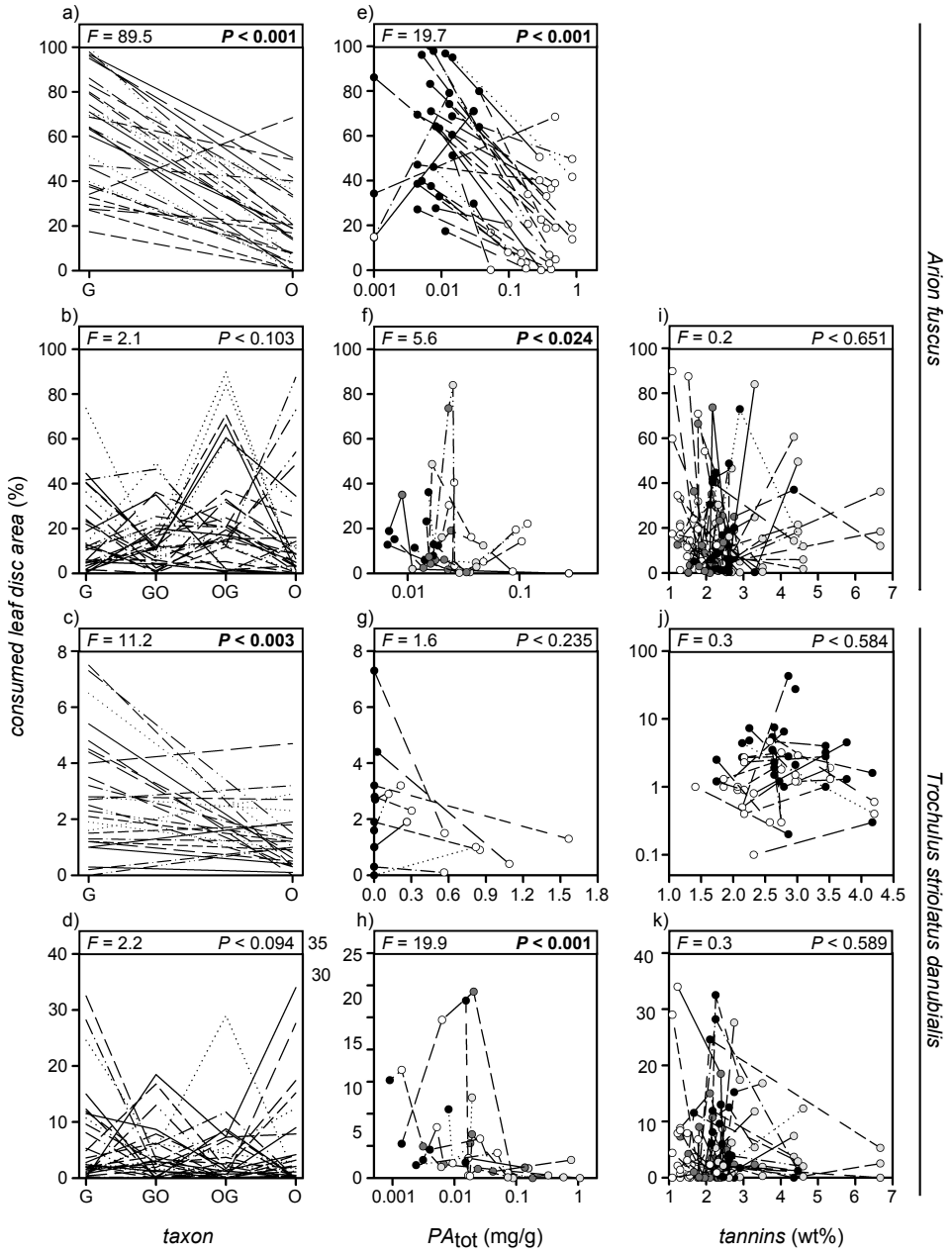

Figure 4.4: Results of the food choice experiments for the *S. germanicus-S. ovatus* system. Leaf discs that were together in the same Petri dish are joint by a line (paired samples). Teststatistics and *P*-values are given above the plots. black circle – G; dark grey circle – GO; light grey circle – OG; white circle – O. Abbreviations: G – *S.germanicus*; GO – *S. germanicus* × *S. ovatus*; OG – *S.ovatus* × *S.germanicus*; O – *S.ovatus.* The first mentioned species was mother plant.

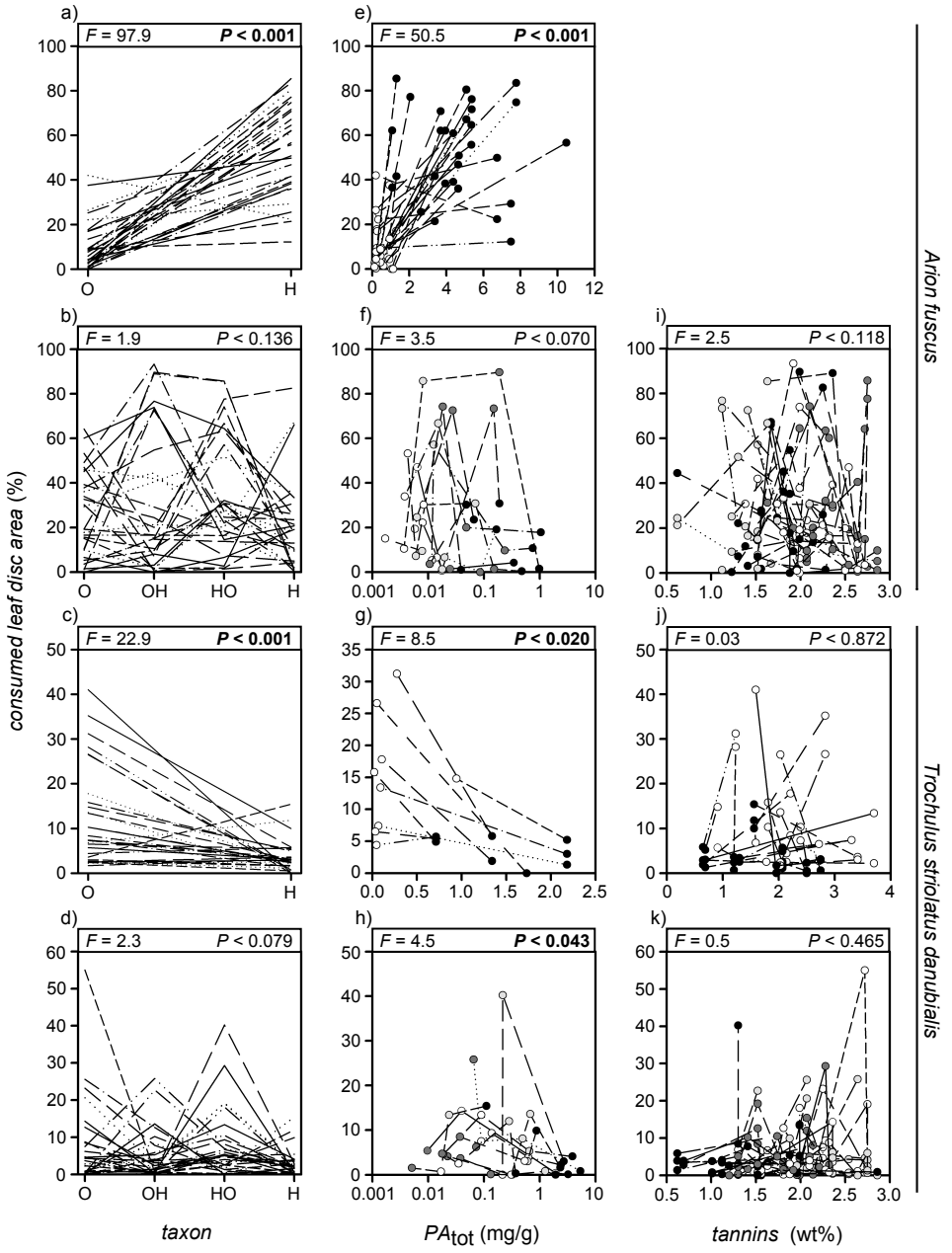

Figure 4.5: Results of the food choice experiments for the *S hercynicus-S. ovatus* system. Leaf discs that were together in the same Petri dish are joint by a line (paired samples). Teststatistics and *P*-values are given above the plots. black circle – H; dark grey circle – HO; light grey circle – OH; white circle – O. Abbreviations: H – *S. hercynicus*; HO – *S. hercynicus* × *S. ovatus*; OH – *S. ovatus* × *S. hercynicus*. The first mentioned species was mother plant.

but *S. germanicus* was mainly characterised by nemorensine and the PA-Mix peak. Moreover, population G2 was characterised by neoplatyphylline. All other peaks occurred sporadically or were lacking at all. According to Herborg (1987), *S. ovatus* contains only PAs with a saturated necine base moiety, but we also found unsaturated PAs, especially in population O2. *Hagers Handbuch der Pharmazeutischen Praxis* (Hänsel et al., 1994) distinguishes among different chemotypes in *S. ovatus* that contain mainly saturated and only small amounts of unsaturated PAs. The presence of different PA chemotypes within several *Senecio* species, and therefore a high intraspecific PA diversity, is also known from other studies (Hartmann and Dierich, 1998; Macel et al., 2004; Pelser et al., 2005; Borstel et al., 1989; Witte et al., 1992), which shows an important characteristic of secondary metabolism and is in line with our findings for the investigated two populations of *S. germanicus* and *S. ovatus*, respectively.

There were also significant differences in tannin content among the three species. *Senecio germanicus* and *S. ovatus* had a significantly higher tannin content than *S. hercynicus*, although these values are very low in comparison to other species that are known for their tannin content and are partially used in the production of leather e.g., *Castanea vesca*: 8 to 14 wt%, *Salix sp.*: c. 10 wt%, *Quercus pedunculata*: 4 to 13 to 16 wt%, *Schinopsis lorentzii*: 6 to 8 wt%, and *Rhizophora mangle*: 15 to 42 wt% (Hölzl and Bancher, 1965).

4.5.2 Effect of hybridisation on phytochemistry

Secondary metabolites usually have a qualitative dominant inheritance mode, as already reviewed by Orians (2000). Thus, it is not surprising that the hybrids investigated in the present study almost always produced the PAs which could be found in the parental species (additive pattern). This finding was also observed by Cheng et al. (2011c), who investigated the PA variation in hybrids of *Jacobaea vulgaris* and *J. aquatica*, another well-studied system within the Senecioneae. Interestingly, we found a novel compound (1,2-dihydro-PA), which was expressed in the two *S. germanicus-S. ovatus* hybrid classes (GO, OG), but was neither found in the one nor the other parental species. However, this compound was present in *S. hercynicus* and its two hybrid classes (HO, OH). To infer the phylogenetic relationship of this three species from their PA composition is not advisable, as Langel et al. (2011) found that closely related species can be very different in their PA patterns. According to Orians (2000), there are at least three mechanisms leading to the presence of novel compounds or to the lack of others. Firstly, biosynthetic pathways could be blocked at a certain point, and intermediate metabolites may accumulate, while end products disappear simultaneously. Secondly, biosynthetic pathways may also be combined (McKey, 1980). Hybridisation establishes such a basis for the

merging of pathways and subsequently, compounds from the parents may be found as partial structures of new compounds, or enzymes from one parental genome process compounds stemming from the pathway of the other parent. This requires the parental pathways being very similar, so that the enzymes are capable of modifying certain compounds. Thirdly, regulatory genes may be disrupted following hybridisation, which could cause a shift in production sites, as demonstrated in *Helianthus*, where a particular sesquiterpene lactone was produced only in the flowers of *H. petiolaris*, but was present in the leaves of the *H. petiolaris* × *H. annuus hybrids* (Orians, 2000). Which mechanism may have led to the formation of the novel compound from GO and OG cannot be determined here, because we do not have additional information about PAs from other parts of the plants (i.e., flowers and roots).

Connected to hybridisation, the ability to produce certain compounds can be transferred from one species to another through introgression (Harborne and Turner, 1984; Hardig et al., 2000). This process is more likely, when the traits are under dominant control or are overexpressed. Additive traits (intermediate content) or traits with incomplete dominance are unlikely to introgress because with every subsequent backcross one would expect a decline in SM content (Orians, 2000). On the basis of our data, we can only speculate about quantitative inheritance or transfer of SMs between species. Although none of the hybrid taxa showed dominance or overexpression in PA content, introgression cannot be fully excluded. It is well known that PAs are produced in the roots of the plants and transported to the shoots and flowers through the phloem (Hartmann and Toppel, 1987; Hartmann et al., 1989). As shoot:root ratio seems to negatively correlate with PA content (Schaffner et al., 2003), we assume the PA content of the measured hybrid classes to be underestimated, because age distribution was not equal in hybrid and parental taxa leading to a bias of the results (see chapter 4.3.4).

4.5.3 Effect of phytochemistry on herbivory

The effect of the two investigated compound classes as antifeedants against mollusc herbivores could only partly be proven for pyrrolizidine alkaloid content and was not demonstrated for tannin content at all. In food choice experiments with two leaf discs offered, the slug *Arion fuscus* preferred in the first experimental design (*S. germanicus* against *S. ovatus*) the species with the lower PA_{tot} and in a second design (*S. hercynicus* against *S. ovatus*) the species with the higher PA_{tot}. The latter experimental design was already tested with the slug *Arion lusitanicus* by Scheidel and Bruelheide (2001), who found a preference for the high-PA_{tot}-containing *S. hercynicus*, too. This argues for a non-deterrent effect of the PAs to *A. lusitanicus* and *A. fuscus*.

A more detailed investigation of the influence of toxic alkaloids on the slug *A. lusitanicus* was done by Aguiar and Wink (2005). They found that the LD_{50} value was 10 to 20 times higher in *A. lusitanicus* than in vertebrates. The least toxic alkaloid of the tested ones was the PA senecionine with an LD_{50} value of $> 1000\,mg\,kg^{-1}$ bodyweight. For the slug it was possible to detoxify the alkaloids very rapidly presumably by the mixed function oxidase (MFO) system or to a lesser extent by segregation via mucus. This shows that molluscs may cope with toxic phytochemicals, quite well. Assuming that the *Arion* species we used in the present study is able to handle the PAs in the same way, we conclude that other taxon-specific features than PAs might be a reason for the observed selective feeding of *A. fuscus*. While mechanical factors (e.g., leaf hairiness or epidermal cell wall thickness) showed limited correlations with consumed leaf area (Scheidel and Bruelheide, 1999b), other chemical features, like sesquiterpenes or the nutrition content, might be considered for the selective feeding pattern of *A. fuscus*. In several *Senecio* species, sesquiterpenes especially from the eremophilane-type, were reported by Fei et al. (2007) and Yang et al. (2011). Additionally, Speiser et al. (1992) could show that sesquiterpenes, in comparison to PAs, were strongly deterrent to the snail species *Arianta arbustorum*. They conclude from their study that PAs are responsible for the toxicity and sesquiterpenes cause the deterrence of a plant. Additionally, they postulate that PAs are protective against vertebrate herbivores while sesquiterpenes are protective against invertebrate ones. The reduced food consumption by *Arianta* as answer to sesquiterpenes was confirmed by Hägele et al. (1996, 1998).

Additionally, the role of nutrition content seems an interesting point. As one would expect, herbivores should prefer individuals with higher nutritional value. Fast growing plant species often have high photosynthetic capacities and high nitrogen content (Mattson Jr, 1980; Mooney and Gulmon, 1982). This could explain the preferred consumption of *S. hercynicus* by *A. fuscus*, as this plant species is adapted to higher elevations, correspondingly shorter vegetation periods, and therefore has faster growth rates than *S. ovatus* and *S. germanicus* (Herborg, 1987). However, there are studies, e.g., by Orians et al. (2013), in which the slug species *A. subfuscus*, a close relative of *A. fuscus*, contrary to expectations, did not select for a lower SM content, but for lower photosynthetical and nutritional features.

In the two-disc experiments, the snail *Trochulus striolatus danubialis* preferred always that species with the lower PA_{tot}, which makes a deterrent effect of PAs conceivable in that case, although there was no significant negative relationship of PA_{tot} and consumed leaf disc area for the experimental design *S. germanicus* against *S. ovatus*. Additonally, the consumption was in general lower for *Trochulus* than for *Arion* leading to the assumption that for different

mollusc species the effect of PA_{tot} is different, too. This was proven for different mollusc populations of the same species, where individuals show adaptations to occurring food plants and their SM content (Speiser et al., 1992).

Tannin concentrations showed no significant influence on the consumed leaf area. This is not surprising, as the tannin content of other plant species is much higher. Fritz et al. (2001) found significant deterrent effects for tannins at concentrations between $50\,mg\,g^{-1}$ to $100\,mg\,g^{-1}$ (5 to 10 wt%), as it can be measured in adult *Salix* species. Therefore, we assume that the tannin concentration found in *Senecio* was too low to be deterrent for the molluscs under study.

4.5.4 Effect of hybridisation on herbivore resistance

Both mollusc species did not show a preference of a certain taxon when there was a choice among four different leaf discs (i.e., parental taxa and reciprocal hybrids). This leads to the assumption that hybridisation may be advantageous for the taxon previously preferred by herbivores. Because hybrids show a 'no difference' pattern according to herbivory, the hybrids-as-sinks hypothesis is unlikely for both investigated hybrid systems. A collision and genetic fusion of the pure species is neither likely, because of the lacking higher hybrid resistance in comparison to the parental species. According to the food choice results, we would expect stable hybrid swarms in the field, where survival is more dependent on other factors (e.g., climate, pollinators, other phytophage species) than mollusc herbivory. Consistent with this, Oberprieler et al. (2010, 2011) found a highly introgressed hybrid swarm in the *S. hercynicus-S. ovatus* system and an asymmetrically introgressed hybrid swarm in the *S. germanicus-S. ovatus* system. This suggests that mollusc herbivory is not the main driver for the outcome of hybridisation, but that there are also other important environmental factors that need to be taken into account.

4.5.5 Conclusions and outlook

Cafeteria experiments are useful instruments to investigate selective consumption of different plant taxa by particular herbivore species (i.e. molluscs). In the present study, we found that the deterrent effect of pyrrolizidine alkaloids, which was proven for some insect taxa is not readily transferable to molluscs, and furthermore that not all molluscs react the same way to these compounds. Some molluscs seem to be deterred by PAs (i.e., *Trochulus striolatus danubialis*), while others are not influenced at all (i.e., *Arion fuscus*). However, we observed a taxon-specific preference, when pure species were tested against each other, which needs to be investigated in further detail. Starting-points

for further analyses could be also sesquiterpenes or the nutrient content of the different taxa. Tannins seem to have no effect on the consumption in the experiments conducted. When four leaf discs were offered to the molluscs, the selective consumption disappeared, which is in contrast to observations by Oberprieler et al. (submitted) and Bog et al. (submitted; chapter 3).

Although cafeteria experiments give a first impression on plant-herbivore interactions, one needs to be cautious with the transfer of lab results to processes in the field. Conclusions drawn from cafeteria experiments cannot be generalised for the fitness or survival of different plant taxa in natural hybrid swarms. In the field, further environmental factors are responsible for the trajectories of a hybrid swarm and may alter the observations that have previously been made in the lab or common garden (Fritz et al., 1999). These factors include for example the composition of herbivore communities, competition with other plant species and several other abiotic factors as well. Since some plants are additionally able to compensate for negative impacts, this makes long-term studies indispensable, in which overall fitness components like pollen fertility, seed set, germination rates, and seedling survival, etc., are monitored.

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CHAPTER 5

General Discussion

Hybridisation is a common phenomenon in the *Senecio nemorensis* syngameon and its occurrence was already proven by Oberprieler (1994) and Oberprieler et al. (2010, 2011, 2015) in the two hybrid systems (i.e., the *S. germanicus-S. ovatus* system and the *S. hercynicus-S. ovatus* system) that are in the focus of the present dissertation. While in the first system hybridisation was rare, and except for a single population the two species mainly formed distinct entities (Oberprieler et al., 2011), in the second hybrid system all examined populations along an elevational transect were found as being hybrid swarms (Oberprieler et al., 2015). This resulted in the questions whether there are eco-climatologically mediated selection regimes acting that lead to the observed patterns. This is the starting point for the present contribution.

In the second chapter, it was shown that most individuals of 38 populations along four elevational transects in the Bavarian Forest National Park (Bavaria, Germany) could be assigned to the *S. ovatus* backcross class indicating advanced introgressive hybridisation in the *S. hercynicus-S. ovatus* system in that region. Several loci were correlated with climatic variables concerning precipitation and/or temperature. This could lead to the assumption that there is climate-mediated selection acting along the transects. However, the selection seems not strong enough to maintain both biological species. On the other hand, loci that mediate climatic adaptations, like temperature and drought stress or shorter development time at higher elevations, might be barely coadapted to loci that play a role in reproductive isolation or these coadaptations were broken up, again (Wu, 2001). Loci that are advantageous are easily transferred among species (Baack and Rieseberg, 2007) and therefore leading to blurred species boundaries. In addition to the question whether there is selection acting along the elevational transects that could maintain

the two species, it is essential to know about the relative fitness of parental species and hybrids (Abbott and Brennan, 2014). In future studies, one should focus on the reasons for the asymmetrical introgression. This necessitates a comparison of pollen fertility, seed set and germination rates of different hybrid classes (i.e., F_1, F_2 and backcross to parentals) with the parental genotypes. Although, pollen fertility was already measured by Hodálová and Kochjarová (2006) and Oberprieler et al. (submitted), these investigations were not done on individuals of known genetic constitution and therefore represent only a little contribution concerning hybrid fitness.

In the third chapter, it was shown that *S. hercynicus* was stronger damaged by herbivores than *S. ovatus*, *S. germanicus* or their reciprocal F_1 hybrids in a transplantation experiment along an elevational transect in the Bavarian Forest National Park. In addition, photosynthetically active and withered leaf area as well as rhizome dry weight were measured. There was evidence for a higher susceptibility of *S. ovatus* compared to *S. hercynicus* and *S. germanicus*. However, it has to be taken into account that the experiment was done only in a single vegetation period. *Senecio germanicus* performed well along the whole transect leading to the question whether this species would manage to produce ripe seeds by the end of the vegetation period in higher elevations, what is indispensable for a successful reproduction and establishment. Hybrid individuals from both hybrid systems showed intermediate values compared to their parental species. A long term reciprocal transplantation experiment with parental and hybrid genotypes could estimate survival over several vegetation periods and also reproduction success measured as ripe seed set of the different taxa including annual climatic fluctuations. Additional caging of individuals would prevent a possible effect of herbivore damage, as it was found that a high herbivore pressure leads to a lower rhizome dry weight and therefore might influence the fitness and survival of the plants.

In the fourth chapter, it was shown that the susceptibility of the plant taxa depended on the mollusc species in cafeteria experiments and that there was an effect of the number of leaf discs offered to the herbivore. *Senecio hercynicus* suffered higher damage than *S. ovatus* by the slug species *Arion fuscus*, but not by the tested snail species *Trochulus striolatus danubialis*. The results show that cafeteria experiments are useful tools to investigate selective consumption of different plant taxa by particular herbivore species, but they are conducted under controlled lab conditions and therefore represent only a small section of the ecological relationships in a natural system. On the other hand, field experiments are less controlled (e.g., climate fluctuations, herbivore communities), but the processes can be observed over a longer time period.

The population genomic study as well as the transplantation experiment and to some extent the cafeteria experiments showed that *S. hercynicus* is in

an inferior position to *S. ovatus* and that neither a climate-mediated selection regime, nor an advantage from herbivore resistance will prevent the genetic swamping of *S. hercynicus* by its congener *S. ovatus* in the Bavarian Forest National Park. Moreover, climate change and a predicted upwards shift of species to higher elevations (Skov and Svenning, 2004; Thuiller et al., 2005) could even strengthen this process. Additionally, introgressive hybridisation of the more herbivore resistant *S. ovatus* with potentially adaptive alleles from *S. hercynicus* is probably an important mechanism leading to a successful establishment of the *S. ovatus* backcross individuals.

The observed patterns in natural populations of the *S. germanicus-S. ovatus* system cannot be exclusively explained by herbivory-mediated selection. Investigations of fitness parameters (i.e., pollen fertility, seed set, germination rate, long term survival) for parental species and several hybrid classes as suggested for the *S. hercynicus-S. ovatus* system would be appropriate to explain the asymmetrical introgression pattern in that system, too. Differences in phenology are not suggested as a good isolation mechanism, as the flowering times of the two species are broadly overlapping (Oberprieler et al., 2011; pers. obs.). The investigation of an eco-climatologically gradient within the distributional limits of *S. germanicus,* reaching its westernmost edge of distribution in south eastern Germany, and *S. ovatus,* reaching its easternmost edge of distribution in the Western Carpathians (Hodálová and Marhold, 1996), could provide insight into a climate-mediated selection regime acting in the maintenance of the two species.

To finally conclude, the present dissertation delivers new insights into the consequences of hybridisation in both hybrid systems under study and simultaneously raises new questions and delivers new starting points for future research.

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APPENDIX A

Supplementary Content

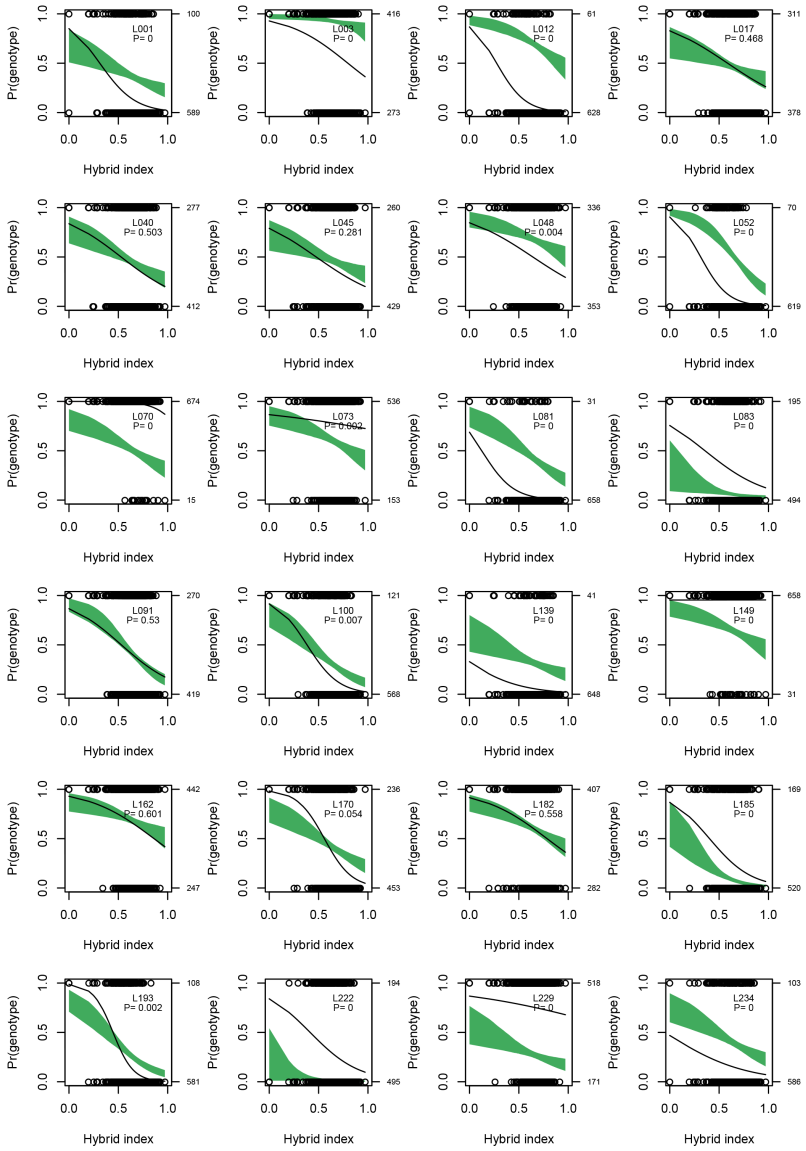

Figure A.1: Fitted genomic clines for the selected 59 loci. Solid line: probability of *S. hercynicus* genotype as function of hybrid index; green area: neutral model; circles: individuals with *S. hercynicus* genotype on top line and individuals with *S. ovatus* genotype on bottom line with quantity given on the right axis.

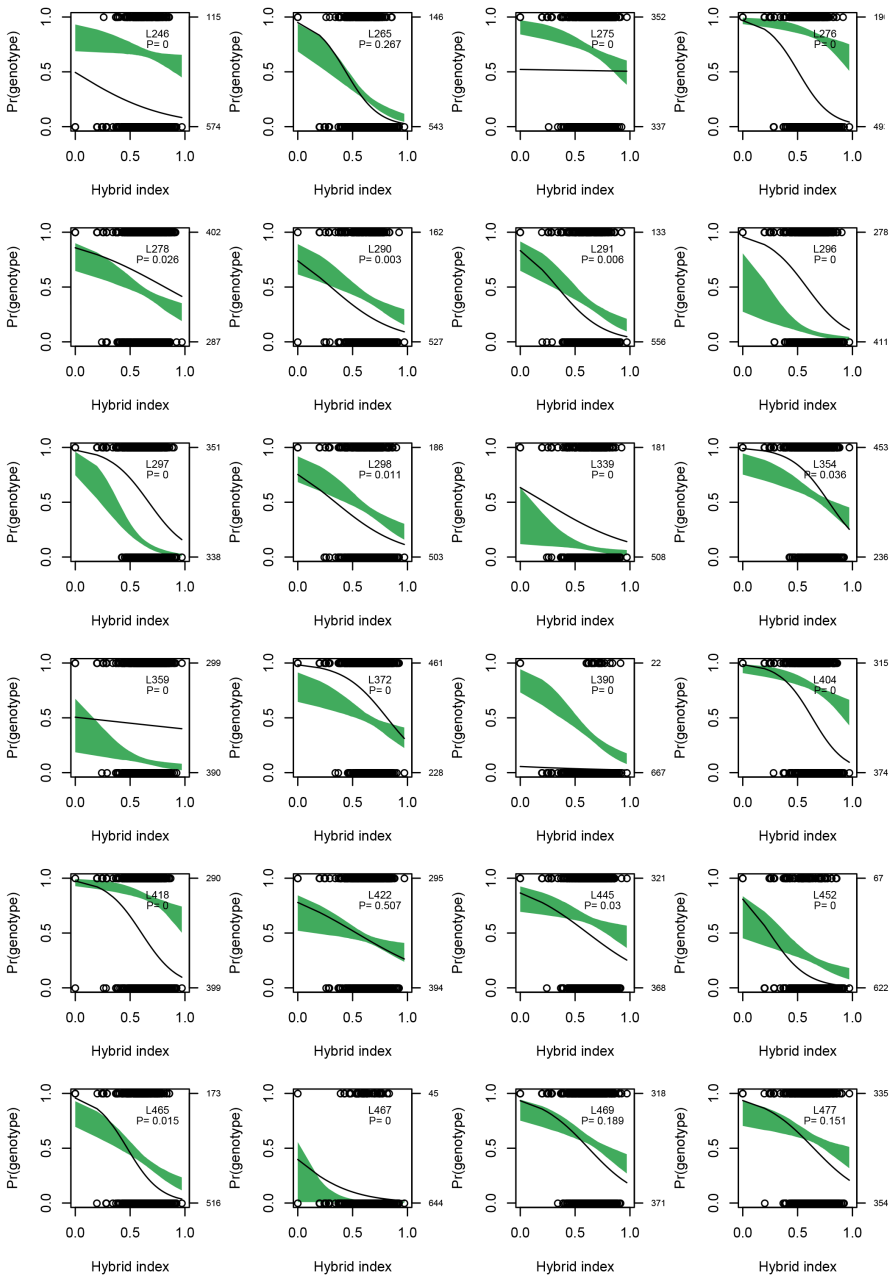

Figure A.1: (continued)

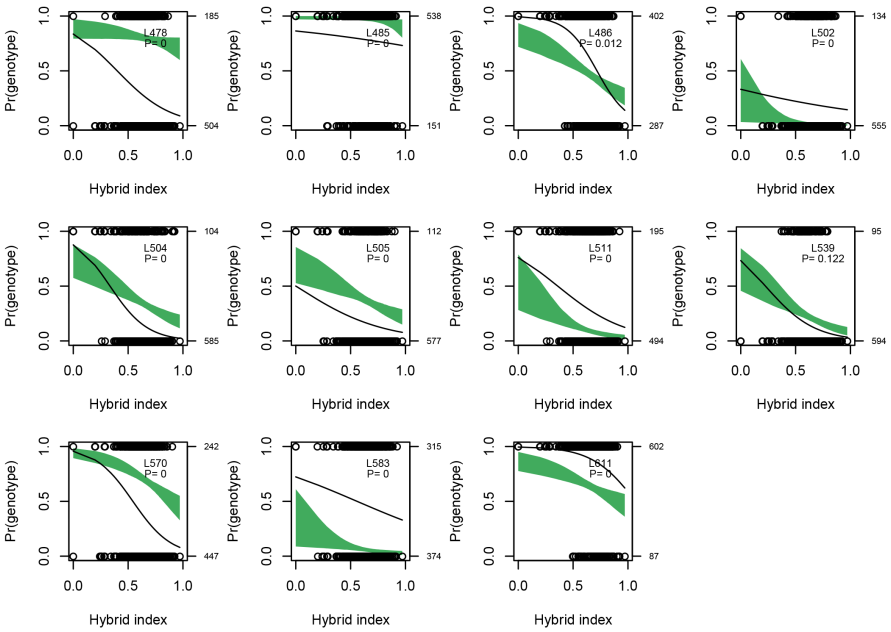

Figure A.1: (continued)

Table A.1: Coordinates, climatic and ecological data for the used plots. T_{year} – mean annual temperature, P_{year} – mean annual precipitation, tl1 – tree layer >15m, tl2 – tree layer <15 m, sl – shrub layer, hl – herb layer

						coverage				
Plot	**Latitude**	**Longitude**	**Elevation**	$\boldsymbol{T}_{\text{year}}$ **(°C)**	$\boldsymbol{P}_{\text{year}}$ **(mm)**	**tl1 (%)**	**tl2 (%)**	**sl (%)**	**hl (%)**	**litter (%)**
P1_1	N48° 46.627'	E12° 56.849'	325 m	8.3	1070	60	30	30	80	60
P1_2	N48° 47.622'	E12° 58.272'	318 m	8.3	1074	30	20	10	30	30
P1_2*	N48° 47.516'	E12° 58.186'	325 m	8.3	1074	60	30	30	80	60
P1_3	N48° 46.770'	E12° 59.868'	317 m	8.3	1070	70	20	30	80	30
P2_1	N48° 50.919'	E13° 22.617'	569 m	6.4	1218	50	15	10	2	40
P2_1*	N48° 50.735'	E13° 22.586'	575 m	6.3	1232	95	0	4	50	80
P2_2	N48° 50.848'	E13° 22.662'	578 m	6.3	1235	95	60	15	20	70
P2_3	N48° 50.761'	E13° 22.602'	540 m	6.4	1217	90	10	0.5	40	30
P3_1	N49° 06.102'	E13° 15.840'	936 m	5.2	1648	90	20	0	5	80
P3_2	N49° 06.056'	E13° 15.818'	914 m	5.4	1636	40	97.5	10	1	97.5
P3_3	N49° 06.064'	E13° 15.752'	894 m	5.4	1629	20	90	30	20	80
P4_1	N49° 06.183'	E13° 17.523'	1173 m	4.4	1621	40	0	5	90	10
P4_2	N49° 06.155'	E13° 17.590'	1197 m	4.3	1610	60	0	10	80	20
P4_3	N49° 06.144'	E13° 17.677'	1216 m	4.2	1604	40	0	10	80	0

* alternative plots in 2012

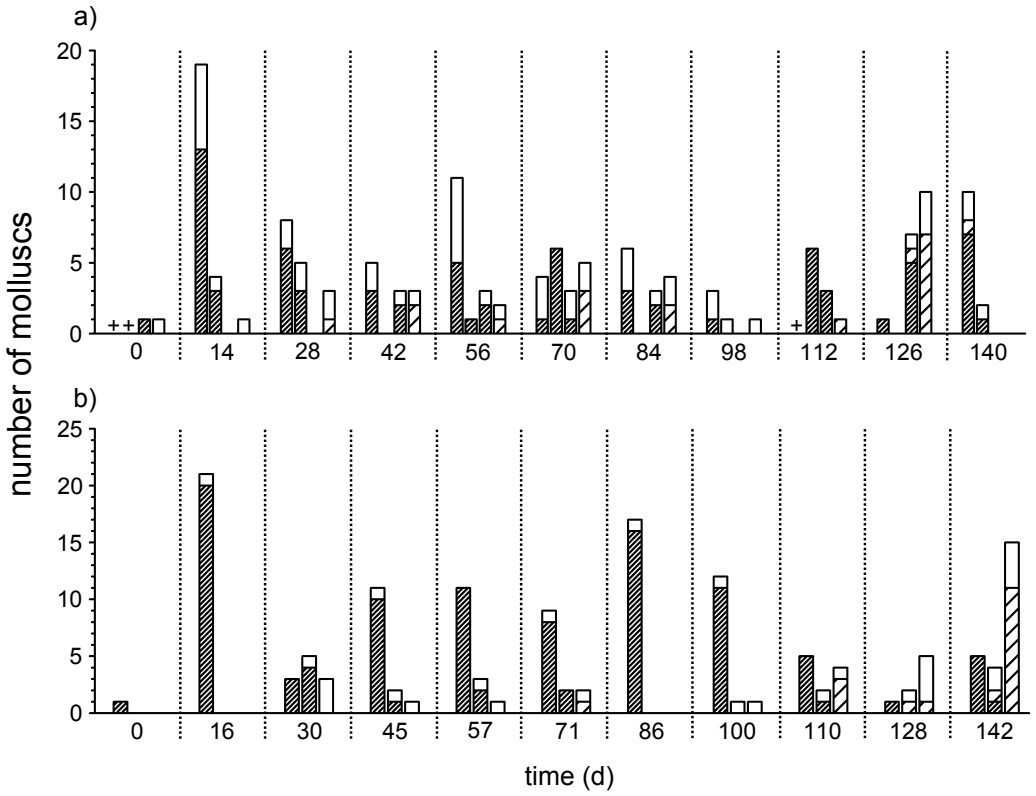

Figure A.2: Number of molluscs and their assignment to one of three mollusc groups over elevation and time. a) 2011; first bar: 300 m, second bar: 600 m, third bar: 900 m, fourth bar: 1200 m, for each date, respectively. b) 2012; first bar: 300 m, second bar: 900 m, third bar: 1200 m, for each date, respectively. hachures – open: slugs, finely dashed: snails, coarsely dashed: semislugs, + value not availabe.

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